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ABSTRACT

Ceratophyllus idius is an ectoparasite of purple martins (Progne subis) and commonly inhabits the nest material of these birds. In the Upper Midwest, purple martins migrate in late August and return in mid- or late April. Despite the host's absence and harsh northern winters, a portion of the adult flea population survives to reinfest returning birds. This study was designed to: (1) examine the possible roles of sorbitol and trehalose as biological antifreezes; (2) confirm the role of glycerol as an antifreeze; (3) monitor glycogen and glycerol levels and super-cooling points from autumn until spring; (4) determine the temperature stimulus triggering glycerol synthesis; (5) determine if glycerol production is reversible; (6) monitor flea survival; and (7) compare sexes in the above mentioned areas.

Assays were done for glycogen, glucose, glycerol, sorbitol and trehalose at 22 C and at 13, 10 and 1 C during a controlled cool-down. After cooling, half of the fleas were held at ambient outdoor conditions and the rest at -6 C. Monthly assays were made mid-October through mid-April on surviving fleas from both regimens.

Glucose, trehalose and sorbitol levels were low in all assays and are not considered important as antifreezes in C. idius. Induction of glycerol synthesis was triggered at or prior to 10 C, and by early winter, glycerol levels were about 25X the time zero value. Concurrently, glycogen

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levels decreased to 6% of the original value in both regimens. After five weeks at -6 C, glycerol levels of freezer-held fleas remained relatively stable through mid-April. Ambient-held glycerol levels varied through mid-April apparently in response to general and short-term temperature trends, and in a controlled warming to 15 C, glycerol levels decreased 26% within two days indicating that the mechanism controlling glycerol levels is both temperature dependent and reversible. In all experiments, glycogen levels varied inversely with glycerol levels. This indicates that glycogen was the major source for glycerol production. In addition, supercooling points corresponded with changing glycerol levels. In the above mentioned assays, the differences between sexes were not statistically significant.

Survivorship for ambient-held fleas generally showed a gradual decline from early September (100%) through mid-April (32.4%) indicating that a substantial portion of the adult flea population is able to successfully overwinter and reinfest purple martins upon their return in April.

ANTIFREEZE PRODUCTION AND COLD-TOLERANCE IN
OVERWINTERING PURPLE MARTIN FLEAS,
CERATOPHYLLUS IDIUS JORDAN AND ROTHSCHILD

Douglas P. Schelhaas, Major, USAF

Doctor of Philosophy

1986

126 pages

University of North Dakota

ANTIFREEZE PRODUCTION AND COLD-TOLERANCE IN

OVERWINTERING PURPLE MARTIN FLEAS,

CERATOPHYLLUS IDIUS JORDAN AND ROTHSCHILD

by
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Bachelor of Arts, Northwestern College, 1967

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A Dissertation

Submitted to the Graduate Faculty

of the

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in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

Grand Forks, North Dakota

August
1986

Ceratophyllus idius is an ectoparasite of purple martins (Progne subis) and inhabits the nest material of these birds. Despite the host's absence (September-April), a portion of the adult flea population survives to reinfest returning birds. This study examined: 1. the effect of temperature on inducing glycerol synthesis; 2. regulation of glycerol levels in response to varying temperatures; 3. supercooling points versus glycerol levels; 4. possible roles of sorbitol, trehalose and glucose as biological antifreezes; 5. glycogen as a source of glycerol; and 5. flea survival.

Assays were done for glucose, glycogen, glycerol and sorbitol during controlled cool-downs, and monthly, mid-October through mid-April, on surviving fleas held at $-6^{\circ}C$ and ambient regimens.

Sorbitol, trehalose and glucose levels were low in all assays and are not considered important as antifreezes in C. idius. Glycerol synthesis was triggered at or prior to $10^{\circ}C$, and by early winter, glycerol levels were about 25X the time zero value. Concurrently, glycogen levels decreased to 6% of the original value in both regimens. After five weeks at $-6^{\circ}C$, glycerol levels of freezer-held fleas remained relatively stable through mid-April. Ambient-held glycerol levels varied through mid-April apparently in response to changing temperatures. In a controlled warmup to $15^{\circ}C$, glycerol levels decreased 26% in two days indicating that the mechanism controlling glycerol levels is both temperature dependent and reversible. In all experiments, glycogen levels varied inversely with glycerol levels. This indicates that glycogen was the major source for glycerol production. In addition, supercooling points corresponded with glycerol levels.

Mid-April, ambient-held survivorship (32.4%) confirmed that adult fleas can successfully overwinter.

Baust, J.G. 1981. Biochemical correlates to cold hardening in insects. Cryobiology 18: 186-198.

Pigage, H.K.; Larson, O.R. 1983. Detection of glycerol in overwintering purple martin fleas, Ceratophyllus idius. Comp. Biochem. Physiol. 75A: 593-596.

Storey, J.M.; Storey, K.B. 1983. Biochemistry of freeze tolerance in terrestrial insects. Trends Biochem. Sci. 8: 242-245.

Zachariassen, K.E. 1985. Physiology of cold tolerance in insects. Physiol. Rev. 65: 799-832.

This Dissertation submitted by Douglas P. Schelhaas in partial fulfillment of the requirements for the Degree of Doctor of Philosophy from the University of North Dakota is hereby approved by the Faculty Advisory Committee under whom the work has been done.

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This dissertation meets the standards for appearance and conforms to the style and format requirements of the Graduate School of the University of North Dakota and is hereby approved.

William Johnson 7/10/86
Dean of the Graduate School

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Title Antifreeze Production and Cold-Tolerance in Overwintering

   Purple Martin Fleas, Ceratophyllus idius Jordan and Rothschild

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Date 1 August 1986

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INTRODUCTION

Numerous insects survive the low winter temperatures of temperate, alpine and polar environments. Since these insects do not migrate or seek warm microhabitats, their survival depends on physiological and biochemical adaptations. An organism's ability to survive prolonged exposure to temperatures below the melting point of its body fluids is called winter-hardiness, cold-hardiness or cold-tolerance (Zachariassen 1985). Various insects accomplish this feat at different stages in their life cycles. Regardless of which stage overwinters, insects belong to one of two distinct categories; (1) freeze-tolerant (insects that can withstand extensive freezing of their extracellular fluids) or (2) freeze-susceptible (insects that die if they freeze). Both situations sometimes occur in the same species, but at different stages of the life cycle (Block 1982; Morrissey and Baust 1976). The situation also can vary from one year to the next in the same developmental stage due to changing environmental conditions (Baust 1981; Duman 1984).

Definitions

Some definitions germane to cold-hardiness are necessary. What constitutes 'cold temperatures' varies among authors. In this dissertation, it will mean temperatures below 0 C, that in the absence of physiological/biochemical mechanisms

1

to produce cold-hardiness, would result in the death of the insect.

The melting point (MP) of a fluid is the temperature at which the last ice crystal in a frozen sample disappears when the fluid is warmed slowly. Conversely, the freezing point (FP) is the temperature at which an ice crystal begins to grow. The MP of pure water is 0 C. Solutes change the physical properties of a solution due to their water binding and colligative properties. These features cause the FP and MP of solutions to drop in proportion to increased solute concentrations (Zachariassen 1985).

Supercooling is the extension of the liquid phase below the freezing point of tissue fluids. The temperature at which spontaneous freezing occurs in such a system is the supercooling point (SCP). The difference between the MP and the SCP is referred to as the supercooling capacity (SCC) of the system.

A proteinaceous antifreeze that prevents the growth of ice crystals below the MP is called a thermal hysteresis protein (THP). In this protective system, the difference between the FP and the MP is termed thermal hysteresis.

When discussing insect cold-hardiness, the term antifreeze often refers to substances which prevent intracellular and extracellular freezing in freeze-susceptible insects. Cryoprotectants usually refer to substances that prevent intracellular freezing, even when extracellular freezing occurs in freeze-tolerant insects.

Freeze-susceptible Insects

Freeze-susceptible species survive by occupying therm-
ally buffered microhabitats or by depressing SCPs of their
body fluids below environmental temperatures by means of
biological antifreezes. These antifreezes include poly-
hydroxyl alcohols (polyols), sugars, THPs and ethylene
glycol. Supercooling points as low as -60 C (Miller and
Werner 1981) and -66 C (Ring and Tesar 1981) are attainable
through the action of these substances. There is generally
a linear relationship between SCPs and antifreeze levels in
freeze-susceptible insects (Block and Young 1979).

The removal or inactivation of ice nucleating agents
(INAs) helps lower the SCP and thus the avoidance of freez-
ing. Freeze-tolerant insects may undergo a slow, controlled
freezing at relatively high sub-zero temperatures due to
the presence of INAs. Figure 1 shows the distribution of
INAs in different categories of cold-hardened insects. In
freeze-susceptible insects the presence of INAs in the gut
and/or body fluids is a serious problem. These must be
removed or inactivated in order to avoid ice nucleation and
death. Several studies have shown that INAs present in the
gut are removed (feeding ceases and the gut is evacuated
naturally) during the cold-hardening process and that this
removal lowers the SCPs for these insects (Block and Zettel
1980; Salt 1953, 1968; Sømme and Conradi-Larsen 1977;
Zachariassen 1980). An increase in free amino acids during
cold-hardening may indicate catabolism of proteinaceous

Figure 1. These diagrams represent the probable distribution of ice nucleating agents in the different fluid compartments of freeze-sensitive and freeze-tolerant insects in summer and winter. o Nucleating agents in the gut or intracellular compartments. ● Nucleating agents in the hemolymph. (Modified from Zachariassen 1980)
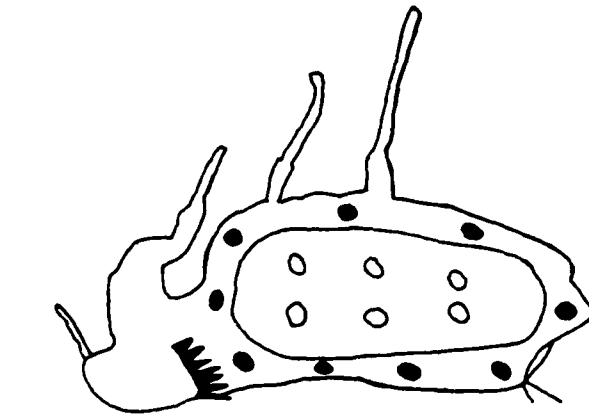
Summer

Winter

Freeze – Sensitive

Freeze – Tolerant
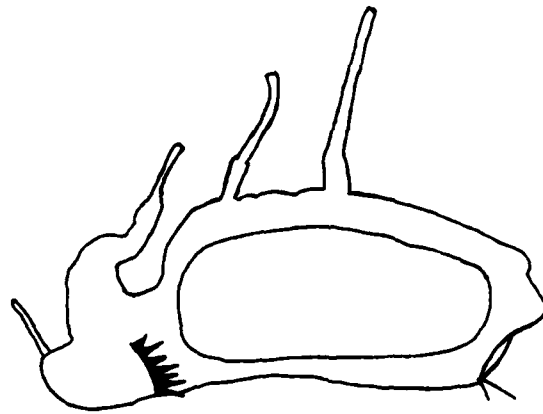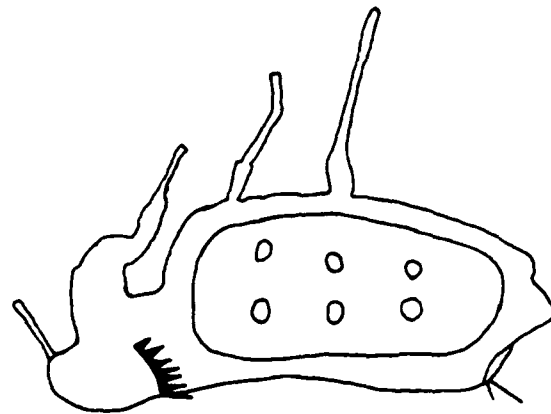
INAs (Sømme 1967; Zachariassen and Påsche 1976). Another process has been suggested for cold-hardened Rhagium inquistor, a freeze-sensitive beetle. This insect has high INA activity associated with membrane structures. This implies that even insects with low SCPs may contain INAs that are sequestered and inactivated within the lipid phase of their cell membranes (Baust and Zachariassen 1983). Baust and Morrissey (1975) reported the possible masking of INAs by polyols and/or sugars, and Duman et al. (1982) proposed that THPs may have the same role.

Freeze-tolerant Insects

In freeze-tolerant insects the SCPs are generally not much depressed. Apparently, INAs in the hemolymph initiate ice nucleation at relatively high sub-zero temperatures (Zachariassen and Hammel 1976; Duman 1982). This is important because supercooling can result in lethal intracellular ice formation even though ice initially forms in the extracellular fluid (Farrant 1980). The development of cold-hardiness in these insects was described by Duman (1982) as follows: "Normally as extracellular ice forms solute is excluded from the ice crystal lattice, thus increasing the osmotic pressure of the unfrozen portion of the extracellular fluid. Consequently, water is drawn osmotically from the intracellular to the extracellular pool, thereby dehydrating the cells. The cell membrane normally prevents seeding of the intracellular fluid by the ice present in the extracellular fluid. As the cells are

dehydrated, the freezing point of the intracellular fluid is depressed, thus decreasing the likehood of intracellular ice formation. However, if the rate of ice crystal growth in the extracellular fluid is high, as would occur if the insect was extensively supercooled prior to freezing, the osmotic flux of water out of the cells may not be sufficient to lower the freezing point of the intracellular fluid to that of the extracellular fluid and consequently lethal ice may form" (p. 622). In addition to protection resulting from dehydration, freeze-tolerant insects may have cryoprotectants to prevent lethal intracellular ice formation (Baust 1973; Storey and Storey 1985).

Three advantages for the early freezing of extracellular fluids in freeze-tolerant insects have been suggested; (1) energy-demanding physiological processes are halted at a higher temperature, thus helping to maintain higher energy reserves (Baust et al. 1979); (2) reduced rates of cell dehydration and a lowered probability of intracellular ice formation are the results of gradual extracellular ice formation (Mazur 1977; Meryman, Williams and Douglas 1977); and (3) biochemical modulation of cryoprotectant levels allows an insect to adjust its levels of cryoprotectants to fit environmental conditions (Baust and Miller, 1970). The effectiveness of this system is shown by freeze-tolerant insects withstanding temperatures as low as -87 C (Miller 1982).

In either method of cold-hardiness, avoidance of

freezing of intracellular fluids is essential for survival.
Beall (1983) stated that there is no measureable crystal-
line solid water in normal functioning cells. Should
freezing occur, possible biological injuries include:
(1) ice crystal growth mechanically rupturing structural
elements; (2) ice acting as a barrier to gas diffusion;
(3) pH changes; (4) denaturation due to altered electro-
lyte concentrations; (5) dehydration causing proteins to
precipitate, or to form abnormal crosslinkages; or
(6) direct removal of structurally important water (Meryman
1966; Ring 1980). Similarities between freeze-tolerant and
freeze-susceptible species include: (1) synthesis of
glycerol or other polyhydroxyl compounds in response to en-
vironmental cues; (2) an ability to supercool (generally
less in freeze-tolerant than in freeze-susceptible insects)
and (3) production of THPs (Duman 1982).

In environments where frequent freeze-thaw cycles occur,
it may be advantageous for insects to be freeze-susceptible
to avoid disruption of their life cycles by a freeze-
tolerant mechanism. Conversely, the metabolic cost of
cryoprotectant synthesis is probably high, and in other
more thermally stable environments, freeze-tolerance may
be more economical (Block 1982).

Some orders of insects (e.g. Hemiptera, Mecoptera) have
been reported as being only freeze-susceptible, while some
(e.g. Hymenoptera, Orthoptera) appear to be only freeze-
tolerant. Yet others (e.g. Diptera and Coleoptera) utilize

both methods for surviving cold environments. Since there is no clear pattern of distribution, the question as to whether freeze-tolerance offers advantages over freeze-susceptibility remains unanswered. There appears to be insufficient evidence to support a phylogenetic component for evolutionary advantage of one process over the other (Block 1982).

Antifreeze/cryoprotectant Agents

Low Molecular Weight (LMW) Agents

Elevated levels of polyhydroxyl alcohols (polyols) have been found in association with cold-hardiness in a large number of insects. Glycerol, the most common biological antifreeze/cryoprotectant in insects, was first reported for silkworms (Chino 1957), for gall flies (Salt 1957) and for cercropia moths (Wyatt and Kalf 1958). Other polyols found in elevated quantities in cold-tolerant insects are sorbitol (Sømme 1965; Storey and Storey 1983a; Mansingh and Smallman 1972; Meryman 1971), mannitol (Sømme 1969) and ethylene glycol (Gehrken 1984). Polyols that have shown slightly elevated concentrations in cold-hardy insects are threitol (Miller and Smith 1975), arabitol (Block and Zettel 1980), ribitol, xylitol, inositol, rhamnitol, fucitol and erythritol (Danks 1978; Ring 1980). These polyols can occur alone or in combination with glycerol, sugars and/or THFs. Polyols function in freeze-tolerant insects by lowering the freezing point of intracellular water. In freeze-susceptible insects, the same substances

lower the freezing points of both intracellular and extra-
cellular water.

Low molecular weight agents depress the FP and SCPs in a
colligative manner. By accumulation of polyols and LMW
sugars, fluid osmolarity may increase to more than 3,000
mosmol/kg (Gehrken 1984; Miller 1982). In these and other
studies, osmolarity has been used to determine polyol
levels. LMW agents may be divided into two categories:
(1) those able to penetrate cells and (2) those that can
not. Because of its small molecular size, glycerol is able
to penetrate cell membranes. An important feature of gly-
cerol is that even at high concentrations it does not
affect enzyme activity. Other polyols and LMW sugars often
occur with glycerol. Zachariassen (1985) suggested that
glycerol affords cryoprotection without affecting enzyme
activity while other polyols and/or sugars may reduce en-
zyme activity. Therefore, it appears that glycerol only
provides cryoprotection, whereas the others may also in-
fluence metabolic processes.

Another hypothesis of how LMW agents enhance cold-hardi-
ness proposes a masking of nucleation sites in freeze-
susceptible beetles (Baust and Morrissey 1975; 1977).
However, at least in some freeze-tolerant insects, LMW
agents do not mask INAs (Lee, Zacharissen and Baust 1981;
Zachariassen and Hammel 1976; Baust and Zachariassen 1983).

In freeze-tolerant insects, extracellular freezing
stresses the intracellular water/solute balance. Dehydra-

tion and concentration of solutes past a certain point may disrupt metabolism and denature cellular proteins (Ring 1980). To avert these effects and to limit the possibility of intracellular ice formation, freeze-tolerant insects apparently eliminate intracellular bulk water and/or increase intracellular bound water. The ordered nature of bound water prevents it from altering its configuration to that of an ice lattice structure. Bound water forms shells around subcellular structures and macromolecules and is believed to prevent their denaturation. At temperatures below the SCP, water in freeze-tolerant insects may exist in only two forms, extracellular ice and intracellular bound water. Bound water content in freeze-tolerant insects appears to increase in response to increased concentrations of hydrophilic polyols (Storey and Storey 1983b; Storey, Baust and Buescher 1981). Estimates of bound water showed a 328% increase when gall fly larvae, Eurosta solidaginis, were moved from 22 C to -30 C (Baust 1981).

Discussion of the synthesis of LMW agents will be limited to three that were assayed for in this project (glycerol, sorbitol and trehalose). In general, it is unlikely that any one environmental trigger provides the definitive signal to initiate the insect cold-hardening processes (Baust 1982). Regulation of polyol synthesis is an example of temperature being the initial trigger for enzymatic processes. Low temperature alters both the rate and pathways used for carbohydrate catabolism (Storey, Baust and Storey

1981; Wood and Nordin 1980). For example, glycogen phosphorylase in the glycolytic pathway of cold-hardy insects is activated by cold temperatures. This results in a rapid rise of the active enzyme form phosphorylase a and glycogen degradation (Ziegler and Wyatt 1975). In some systems, lowered temperatures do not directly affect enzyme activity, but the ones involved in polyol synthesis (i.e., phosphorylases, phosphofructokinases, dehydrogenases) demonstrate increased activity in cold acclimated insects. A proposed pathway for glycerol and sorbitol synthesis is presented in Figure 2. This pathway shunts triose phosphates from the glycolytic pathway into glycerol synthesis. In the gall fly larvae, E. solidaginis, phosphofructokinase is strongly inhibited at lower temperatures, thus blocking glycerol synthesis at the hexose phosphate level, with sorbitol instead of glycerol being produced (Storey and Storey 1983b).

Trehalose is composed of two glucose units joined in 1,1α linkage. The naturally occurring isomer, α-trehalose is shown below. It is known to occur in many insects.
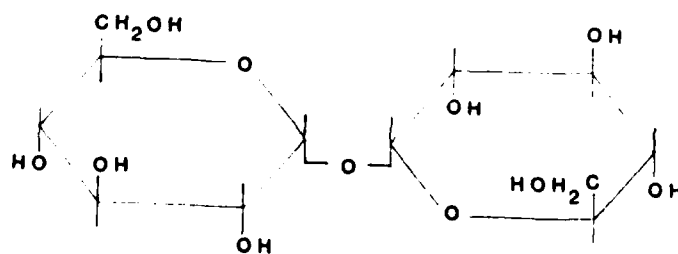
Wyatt (1967) considered it to be the predominate hemolymph sugar in most insects studied, although in some insects it

Figure 2. Proposed pathways for glycerol and sorbitol synthesis in larvae of Eurosta solidaginis. *Phosphofructokinase (PFK) inhibition. (Modified from Storey and Storey 1981)

Glycogen

↓

Glucose-1-P

↓

Fructose-6-P ⟶ Glucose

Fructose-6-P ⟶ Fructose

SORBITOL

*

↓

Fructose-1,6-diP

Dihydroxyacetone-P   Glyceraldehyde-3-P → → → TCA cycle

↓   ↓

Glycerol-3-P   Glyceraldehyde

GLYCEROL
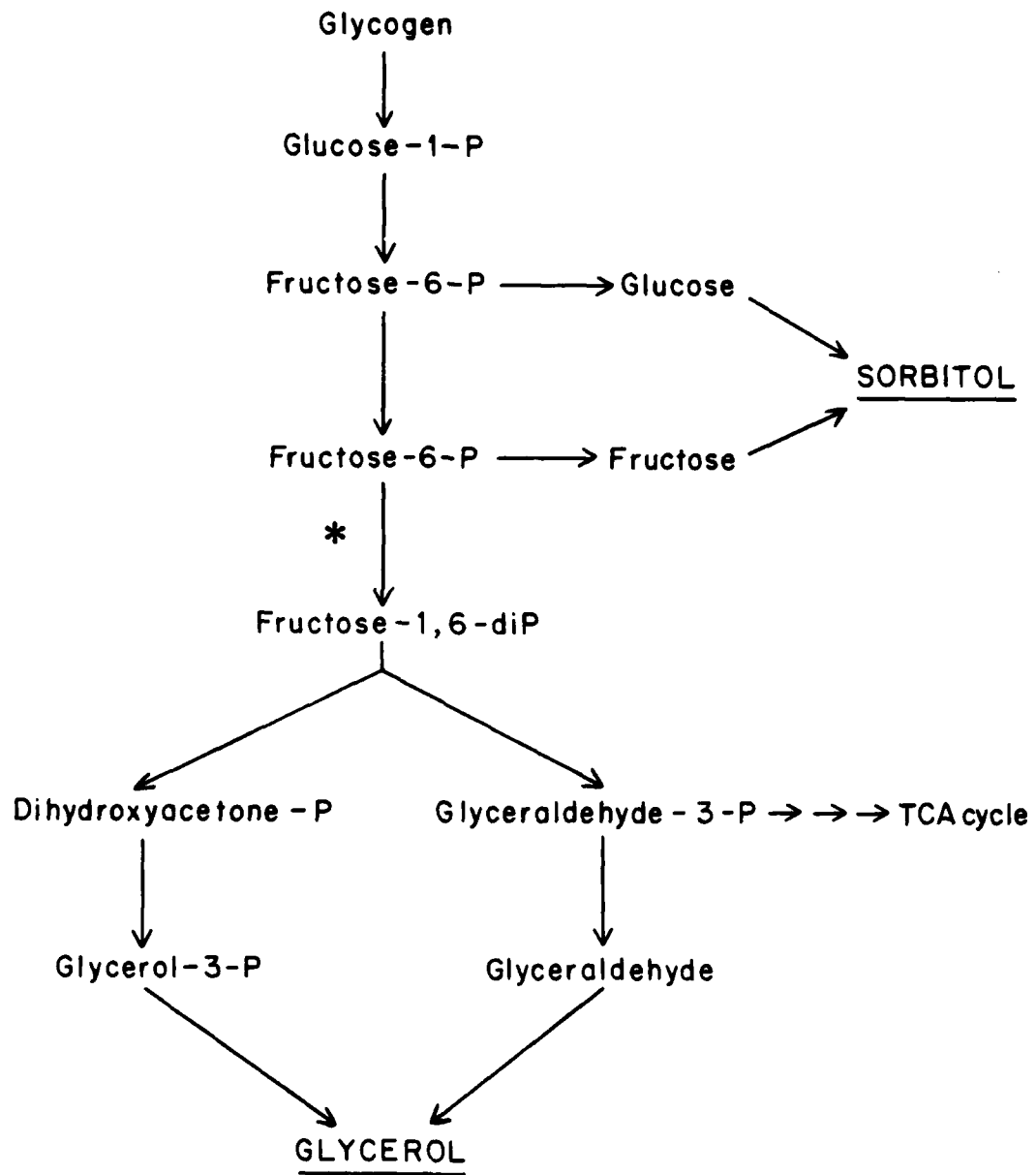
is found only in low concentrations (Rockstein 1978).

The pathway from glycogen to trehalose (Figure 3) involves not only an initial phosphorylation (from inorganic phosphate or ATP), but also a second one from uridine triphosphate. Synthesis of trehalose, therefore, requires an extra energy requiring step. However, an advantage of trehalose over glucose is its nonreactive property. It can be stored in high concentrations in the hemolymph without the risk of promoting undesirable side reactions. It should be noted, however, that some insects possess high levels of reducing sugars in the hemolymph (Gilmour 1965). Two other advantages of trehalose are a lower osmotic effect than from comparable glucose concentrations, and an enhancement of glucose absorption by facilitated diffusion (Rockstein 1978).

Trehalose is metabolized by the enzyme trehalase by the following reactions (Elbein 1974):

$$\text{trehalose-6-P} \longrightarrow \text{trehalose} + P_i$$

$$\text{trehalose} \longrightarrow 2 \text{ D-glucose}$$
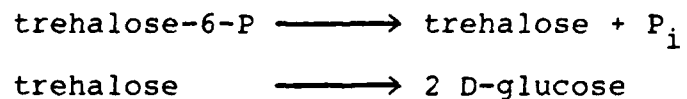
Although most cold tolerant insects produce polyols, similar protection can be provided by trehalose. For example, the Japanese poplar sawfly, Trichiocampus populi, survives liquid nitrogen temperatures without polyols, but does possess very high trehalose levels (Asahina and Tanno 1964; Tanno 1970). Hayakawa and Chino (1981) reported that the silkworm, Philosamia cynthia, overwinters using high concentrations of trehalose. Cold tolerant insects some-

Figure 3. Metabolic pathways linking glucose, trehalose and glycogen in insects. (Modified from Wyatt 1967)
UTP = uridine triphosphate; UDPG = uridine diphosphate glucose; UDP = uridine diphosphate; PP_i = pyrophosphate
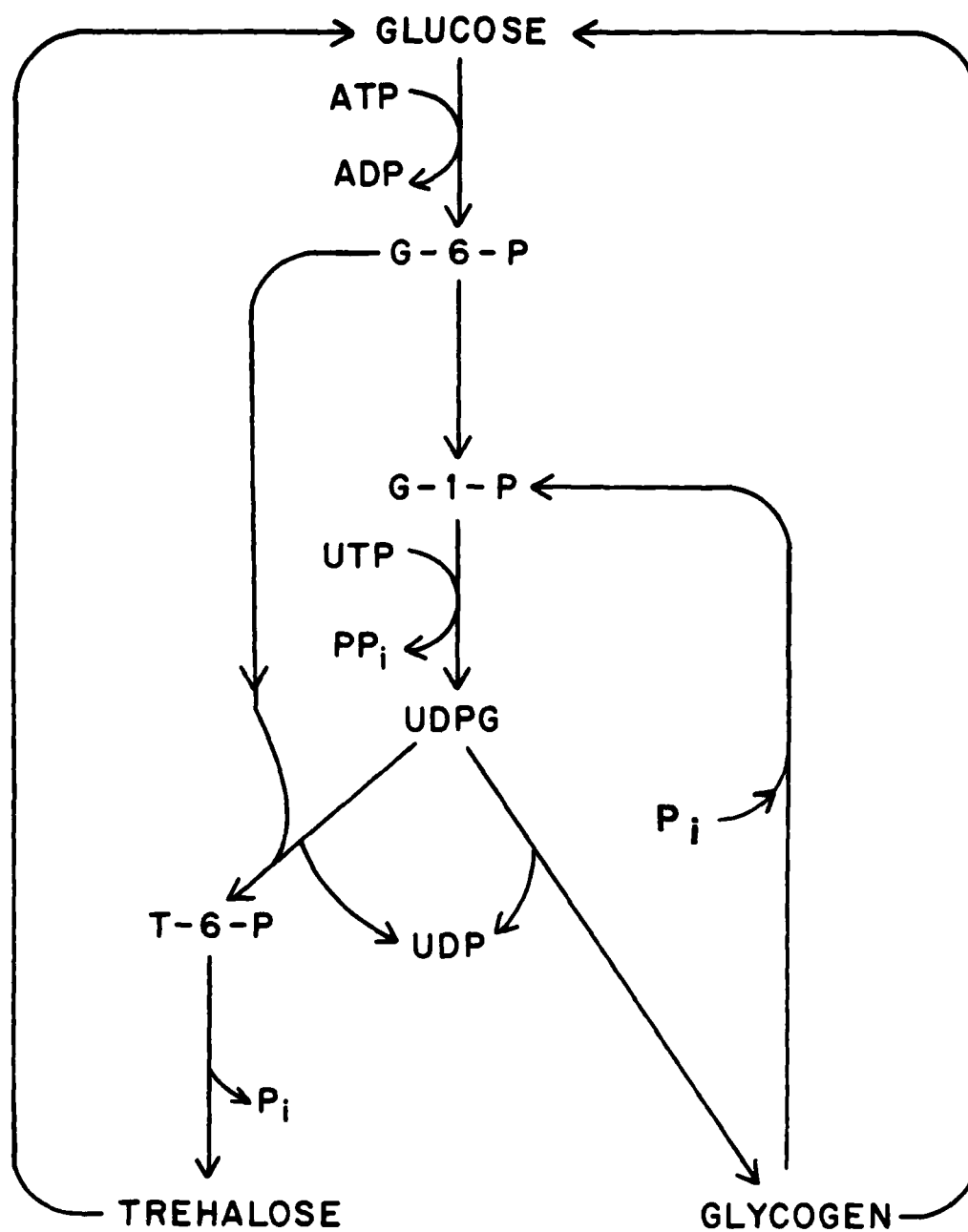
times are divided into two categories, those that accumulate polyols and those that accumulate trehalose (Hayakawa and Chino 1982).

Protein Antifreezes

Duman (1977a;1977b) first reported macromolecular antifreeze agents, thermal hystersis factors (THFs), in overwintering beetles. Since then, THFs have been reported for 20 species of insects (Zachariassen 1985). These protein macromolecules, have been found in every winter-hardy insect tested for THFs, including polyol and non-polyol producers. It may be that THFs are more characteristic of cold-tolerance in insects than are polyols (Zachariassen 1985).

THFs appear to stabilize the supercooled state. Their mechanism of action has not been studied in insects, but in marine teleost fishes, Raymond and DeVries (1977) reported THFs adsorbed to the surface of ice crystals. Growth of such crystals is thought to be restricted to areas between the THF molecules. This results in a highly curved front available for ice crystal growth. Growth between adjacent molecules will stop when the ice front reaches a critically high curvature, and further growth can then take place only at lower temperatures (Zachariassen 1985).

An important advantage of THFs over small molecular weight antifreezes is that they do not function in a colligative manner and therefore do not generate the high osmotic pressures that result from high levels of LMW

agents. Most LMW agents are involved in metabolic pathways. Therefore, maintainance of elevated levels might be difficult during periods of high metabolic activity (Duman et al. 1982). This view appears consistent with the observations of reduced levels of glycerol seen in cold-hardened insects when warmed (Baust 1981). Since THFs are not directly involved in metabolic pathways, insects can synthesize them in early fall and keep them through late spring, thus providing frost protection before and after the occurrence of polyols (Duman 1980). It is speculated that THFs have other, non-antifreeze roles (Duman et al. 1982). THFs occurring in the insect gut would not only prevent intestinal ice formation, but would also prevent inoculative freezing of the hemolymph (Duman 1984). However, since THFs do not penetrate cell membranes, they are unable to prevent intracellular damage when the extracellular fluids of a freeze-tolerant insect freeze (Duman 1977a).

The Purple Martin Flea

Ceratophyllus idius Jordan and Rothschild is an obligatory parasite of tree swallows (Iridoprocne bicolor) and purple martins (Progne subis) in temperate North America and commonly inhabits nest material of these birds. Host ranges are transcontinental and northward to central Alberta (purple martins) and northcentral Alaska (tree swallows) (Terres 1980). It is not known if C. idius occurs or survives in all areas where the hosts nest, but they occur from Alaska, across boreal Canada to

Newfoundland (Holland 1985), and coast to coast in the con-
tiguous United States north of about 40° N latitude (Lewis
1975). In the Upper Midwest, purple martins migrate in
late August and return in mid- or late April, thus separat-
ing the fleas from their hosts for 7.5 to 8 months. Despite
the host's absence and harsh northern winters, some portion
of the adult flea population survives to reinfest birds upon
their return (Larson 1973).

The systematics and public health importance of fleas
have been studied extensively. However, relatively little
is known about the life cycles and behavior of these
insects. Specific information about C. idius is limited,
and what follows is a generalized account of flea biology.

Adult fleas are obligatory parasites, mostly associated
with mammals, and less commonly with birds. Adults are
wingless, sexually dimorphic, laterally compressed and
covered with backward pointing ctenidia and bristles which
contribute to attachment to the host. The hind legs are
modified for jumping. Thus fleas are not easily dislodged,
but have the ability to move great distances rapidly. Many
studies have noted that populations of fleas found on hosts
often fluctuate throughout the year. Such seasonal changes
reflect variations in fecundity, environmental influence on
preadult stages, and climatic or ecological factors that
cause adults to remain in the nest or den of the host.

A generalized life cycle is presented in Figure 4. The
oval, white or ivory colored eggs may be laid in the host's

Figure 4. Generalized flea life cycle.

ADULT

blood feeder   one mating

PUPA (in cocoon)   EGG

LARVA

3 stages

debris feeder
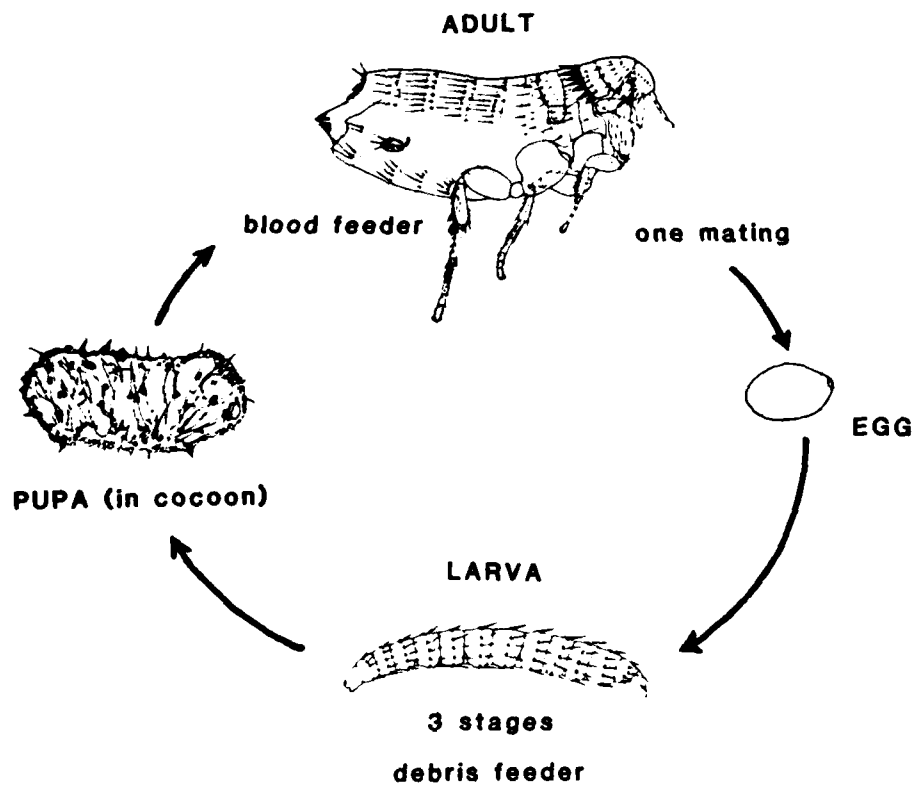
FLEA LIFE CYCLE
one month

feathers or fur, or in the nest material. Larvae are slender, cylindrical and apodous. They feed on dried blood, feces of adult fleas and other organic matter. However, some larvae ingest blood or body fluids via a sucking action (Benton, Surman and Krinsky 1979). The pupal stage is enclosed in a cocoon of silk and nest debris. The time of emergence varies since the adult can remain quiescent after metamorphosis is complete. Mechanical and chemical stimuli may trigger emergence. Birds living in burrows or well protected nests tend to be infested with fleas. Eggs and larvae require high humidity and warm temperatures for development. Pupae and adults are less sensitive to changes in microclimate, and with proper conditions, a typical life cycle requires about 30 days. The average developmental time is about three days for each of the first three stages (egg, first and second instars) and about five days each for the third instar and pupa (Poole and Underhill 1953). Life spans of adult fleas have been reported to range from 158 to 477 days at 20 C and 90-94% relative humidity. Frequency of blood meals was thought to be the most important factor for individual longevity (Burroughs 1953).

Cold tolerance in fleas has received little study. Rothschild (1965) reported that in England the rabbit flea, Spilopsyllus cuniculi, survived at about -1 C in a refrigerator for nine months, but not for prolonged periods at less than -10 C. North American small mammal fleas,

Orchopeas leucopus and O. sexdentatus survived one week to three months at -10 C to -15 C in a freezer, and up to three weeks at ambient temperatures of -10 to -25 C (Miller and Benton 1970). Ctenophthalmus agyrtes agyrtes successfully overwintered in soil at subzero temperatures in Czechoslovakia (Daniel 1973).

Larson (1973) examined the cold-hardiness of C. idius in laboratory and natural conditions. He found adult fleas survived better at -8 C than at 5 C, and that about 90% were still alive after 18 weeks at -8 C. Active adults were noted in late April prior to raising the martin house upon its pole. Therefore, some fleas survived to reinfest birds upon their return. Pigage and Larson (1983) reported that adult fleas survived mean January and February temperatures as low as -20 C, with occasional drops to -30 C, although the survival rate by April was only about 4%. Ponzio and Larson (1984) reported similar results, although the survival rate was higher, about 13%. These reports noted a tendency for higher survival rates for females than for males, and better survival for both sexes at -5 or -6 C than under ambient conditions.

A survey of the literature indicates that the only studies of flea cold-tolerance occurring anywhere, are those conducted at the University of North Dakota since the early 1970s. It also appears that C. idius is the first ectoparasite to have its antifreeze qualitatively and quantitatively analyzed.

Purpose of This Study

This study was designed: (1) to examine the possible roles of sorbitol and trehalose as biological antifreezes; (2) to confirm the role of glycerol as an antifreeze; (3) to compare glycogen and glycerol levels during anti- freeze synthesis; (4) to quantify the levels of these substances from early September through in mid-April; (5) to determine the stimulus triggering glycerol synthesis; (6) to determine if glycerol production is reversible; (7) to determine the effect of a simulated mid-winter thaw on antifreeze levels; (8) to determine SCPs at various points in the cold-hardening process and to correlate such with antifreeze levels; (9) to compare flea survival dur- ing two temperature regimens; and (10) to compare sexes in the above mentioned areas.

MATERIALS AND METHODS

Procurement and Processing of Fleas

Collection

Since one aspect of this study dealt with the induction of cold tolerance, attempts were made to collect nest material from purple martin bird houses as early in the autumn as possible. This was done after birds began their fall migration (bird house owners would not allow earlier cleaning). Nest material was procured from bird houses in the greater Grand Forks, North Dakota area. In 1984, collections were made during 30 August-5 September from 12 houses, and in 1985, during 3-5 September from 10 houses. Bird houses were located via personal referral and through a newspaper advertisement offering free bird house cleaning. Nest material was double bagged in opaque plastic trash bags. These were secured and stored at room temperature (22 C) for 2-8 days until the fleas could be isolated, randomized and packaged.

Isolation

Small amounts of nest material were placed in a white enamel pan having a depth sufficient to keep fleas from jumping out. The mobile, dark brown fleas were easily seen against the white background and aspirated into a collecting vial. Fleas recovered from each house were placed into small, cloth covered jars and held at 22 C until randomized.

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Approximately 4,500 fleas were isolated in 1984 and over 5,500 in 1985.

Randomization

Because of possible differences in winter survival between populations of fleas, the collections were randomized. The number of fleas from a given house which were placed in a vial was proportional to the number of fleas isolated from that house. For example, the 100 vials assembled in 1985 had the following proportions:

Bird House No.	No. of Fleas	No. of Fleas/Vial
1	1000+	10
2	1000+	10
3	1000+	10
4	1000+	10
5	1000+	10
6	500+	5
	5500+	55/vial

In 1984, the procedure was the same except that the numbers were different, with 73 vials receiving 52 fleas each. All vials were covered with prewashed 100% cotton cloth secured by rubber bands. Cotton was used to preclude toxic effects that could have resulted from synthetic fabric.

Weight Loss of Fleas

Prior to beginning the cool-down process, four vials of fleas were freeze-killed in order to determine time zero carbohydrate and antifreeze levels. All fleas were weighed just prior to grinding. This also allowed comparison of

weights between males and females, and the identification
of any vial in which fleas were excessively light due to
freezer dehydration. For comparative purposes, the weight
loss of freeze-killed fleas stored in double-sealed con-
tainers at -25 C was monitored as follows: two vials of
time zero fleas were freeze-killed, weighed, sealed and
stored at -25 C. Vial 1 was opened and weighed at two
months and vial 2 at four months. Vials 1 and 2 were re-
turned to the freezer and weighed again at the next two
month interval.

Cool-Down

In both years, fleas were cooled at a rate of 3 C
every fourth day (Figure 5) until reaching 1 C. This was
done by using two incubator/coolers (General Electric) and
moving the fleas at the proper time interval. After four
days at 1 C, vials were separated into either ambient or
freezer-held regimens. Throughout the cooling process and
all subsequent aspects of the project, fleas were held in
total darkness.

Experimental Temperature Regimens

Freezer-held vials in a light proof box with adequate
ventilation were placed in the freezer compartment of a
refrigerator and maintained at -6 C. Ambient-held vials
were placed in a plastic box (light proof but with adequate
ventilation). This box in turn was placed in a larger wood-
en box on the roof of Starcher Hall at the University of
North Dakota. This box was located on the north side of a

Figure 5. Flow sheet detailing the steps in the cool-down process.
Procedure identical both years, except for added items in 1985.

Time zero   fleas held at   Freeze-killed fleas for
          22 C   time-zero analysis:
                   SCPs for time zero (1985)

Day 4   fleas moved to
          19 C

Day 8   fleas moved to
          16 C

Day 12   fleas moved to
          13 C

Day 16   fleas moved to   Freeze-killed fleas for
          10 C   13 C analysis (1985)

Day 20   fleas moved to   Freeze-killed fleas for
          7 C   10 C analysis:
                   SCPs for 10 C (1985)

Day 24   fleas moved to
          4 C

Day 28   fleas moved to
          1 C

Day 32   fleas divided   Freeze-killed fleas for
        between two   1 C analysis:
         regimens   SCPs for 1 C (1985)

Ambient conditions   Freezer (-6 C)

wall in perpetual shade, and was also elevated on bricks to reduce the effect of thermal conduction from the roof. Temperatures measured on the outer box were known to be within one degree of locally reported ambient temperatures (Ponzio 1984). Daily maximum and minimum temperature readings were obtained from the University of North Dakota Geography Department. In experiments involving a sudden warm-up, vials were placed in the incubator/coolers mentioned above. A standard upright freezer at -25 C was used for storage of freeze-killed fleas. The various temperature regimens used in this project are given in Figures 6 and 7.

Freeze-killing of Fleas

At various times in the cool-down and experimental regimens, fleas were freeze-killed. Fleas that had been held at subzero temperatures were allowed to warm to room temperature in a white enamel pan for 15-20 minutes. Dead fleas were sexed, counted and stored in 70% ethanol. Living fleas were placed in capped glass vials and quick killed in a dry ice/acetone bath (-70 C) for 10 minutes. Live fleas were those showing any sign of life, even twitching tarsi as observed with the aid of a dissecting microscope. After removal from the -70 C bath, each vial was sealed with paraffin, placed in a larger air tight jar and stored at -25 C until the fleas were analyzed.

Assays Procedures

Standards

Standards for the various assays were made by serial

Figure 6. Flow sheet detailing the environmental regimens and sampling dates for fleas during 1984-85.

1 C
4 Days

Ambient   Freezer (-6 C)

Oct 15   Oct 15

Nov 15   Nov 15

Dec 15   Dec 15

Jan 15   mid-monthly   Jan 15*
                freeze-kills
                for analysis of
Feb 15   glucose, glycogen,   Feb 15
                glycerol and
                sorbitol
Mar 15   Mar 15

Apr 15   Apr 15
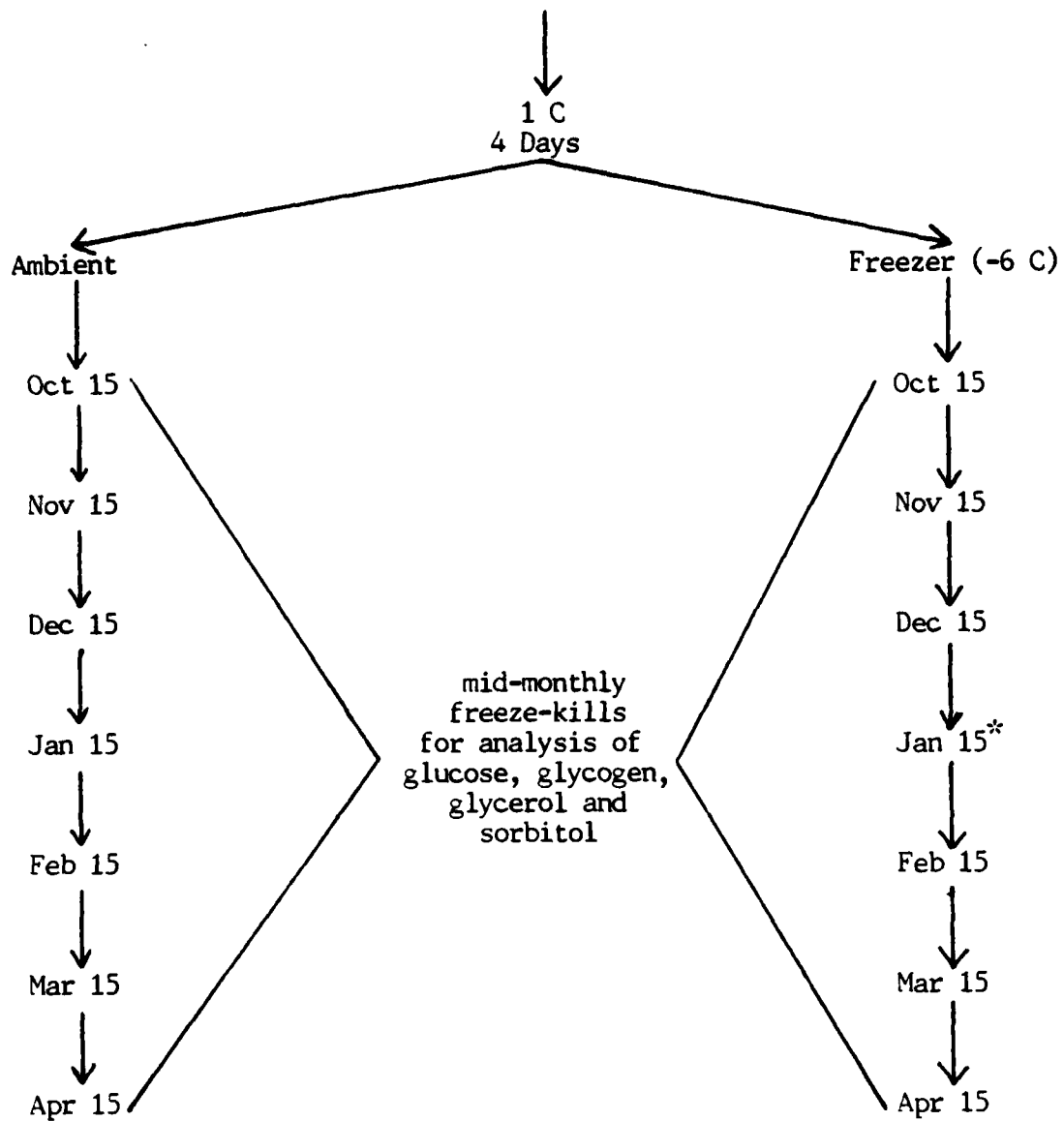
*mid-winter thaw simulation

Sample of fleas moved to
2 C for 7 days, then compared
with January 15 cohorts.

1 C
4 Days

Freezer (-6 C)

20 vials of fleas to test reversibility/temperature dependence of glycerol synthesis

Time-zero --- Oct 22, 1985 fleas moved to 15 C

SCP determinations and freeze-kills for analysis of glucose, glycogen, glycerol and trehalose

Day 2 → Day 4 → Day 8

1 vial recooled to -6 C for 18 days

Oct 22 → Nov 15 → Feb 15 → Mar 15 → Apr 15

SCP determinations and freeze-kills for analysis of glucose, glycogen, glycerol and trehalose

Ambient

Feb 15

Mar 15

Apr 15

SCP determinations and mid-monthly freeze-kills for glucose, glycogen, glycerol and trehalose

dilutions using volumetric flasks to reach the desired concentrations. These were expressed in μg/ml and graphed as standard curves (Figures 8 and 9). Readings for standards and all assays were made with a Beckman DU-7 spectrophotometer. Examples of data collection sheets are presented in Appendices 1-4.

Glucose and Glycogen Assays (Figure 10)

At the beginning of every assay, fleas were sorted by sex and weighed on an analytical balance (Mettler). Analyses were done on groups of 4 to 10 specimens. These were homogenized in 0.5 ml 80% methanol in a tissue grinder (Kemp and Kits van Heijningen 1954). Two methanol rinses, 0.75 ml each, were pipetted into the grinding tube and the pestle run for several seconds. The two rinses were combined with the homogenate and spun in a bench top laboratory centrifuge at 4,000 rpm for five minutes.

Glucose Assay

For this analysis 0.3 ml of supernatant was pipetted into three 10 x 120 mm test tubes (assay tubes). A blank tube received 0.3 ml 80% methanol, and three additional tubes 0.3 ml each of 5 μg/ml glucose standard in 80% methanol. Sigma reagent (0.9 ml) was then added to each tube. All were incubated in a water bath at 37 C for 30 minutes. The optical density (O.D.) was read within 30 minutes on a spectrophotometer at 450 nm. This test is based on the simultaneous use of glucose oxidase and peroxidase coupled with a chromogenic oxygen acceptor,

Figure 8. Standard curves for glucose and trehalose.
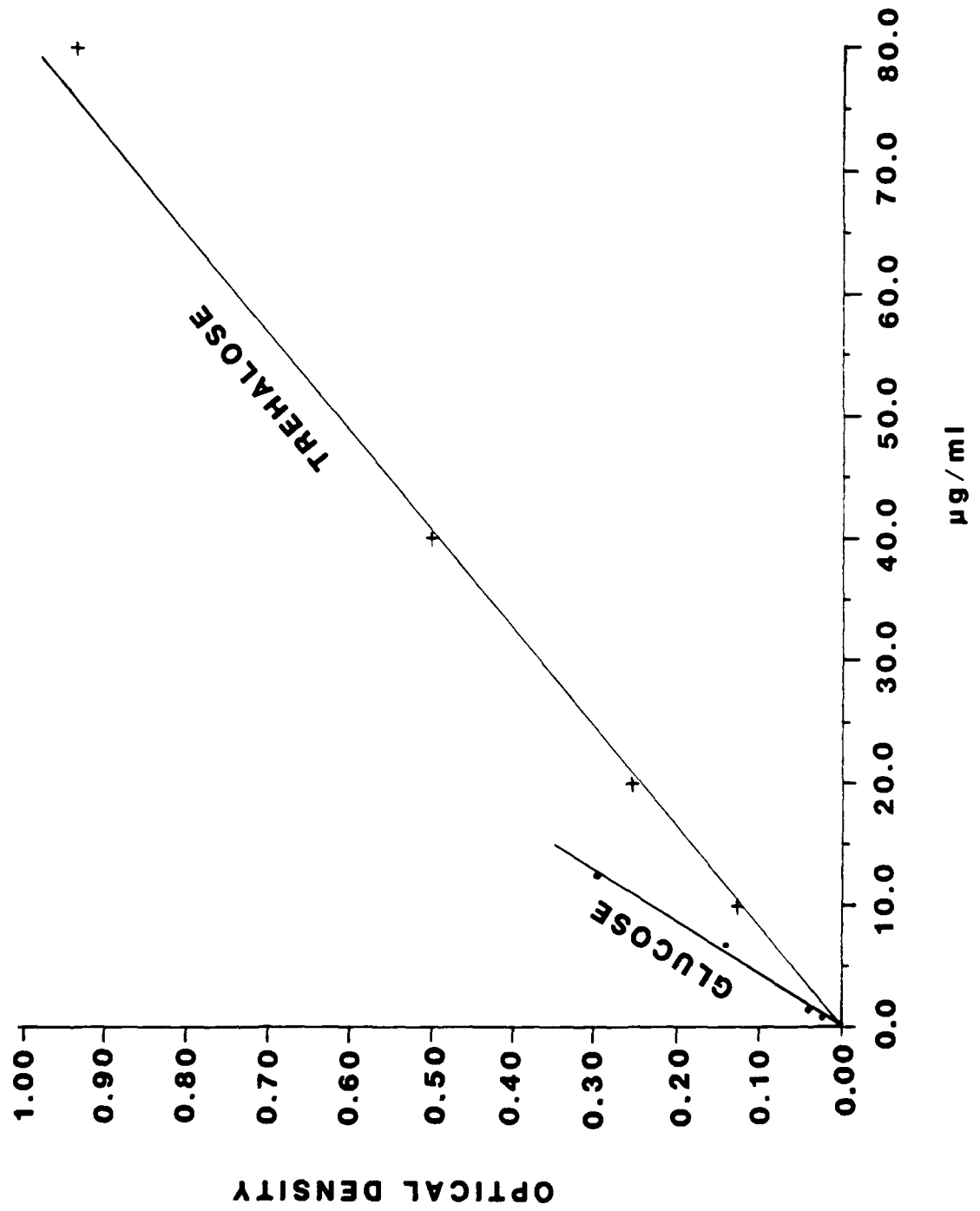
Figure 9. Standard curves for glycerol and sorbitol
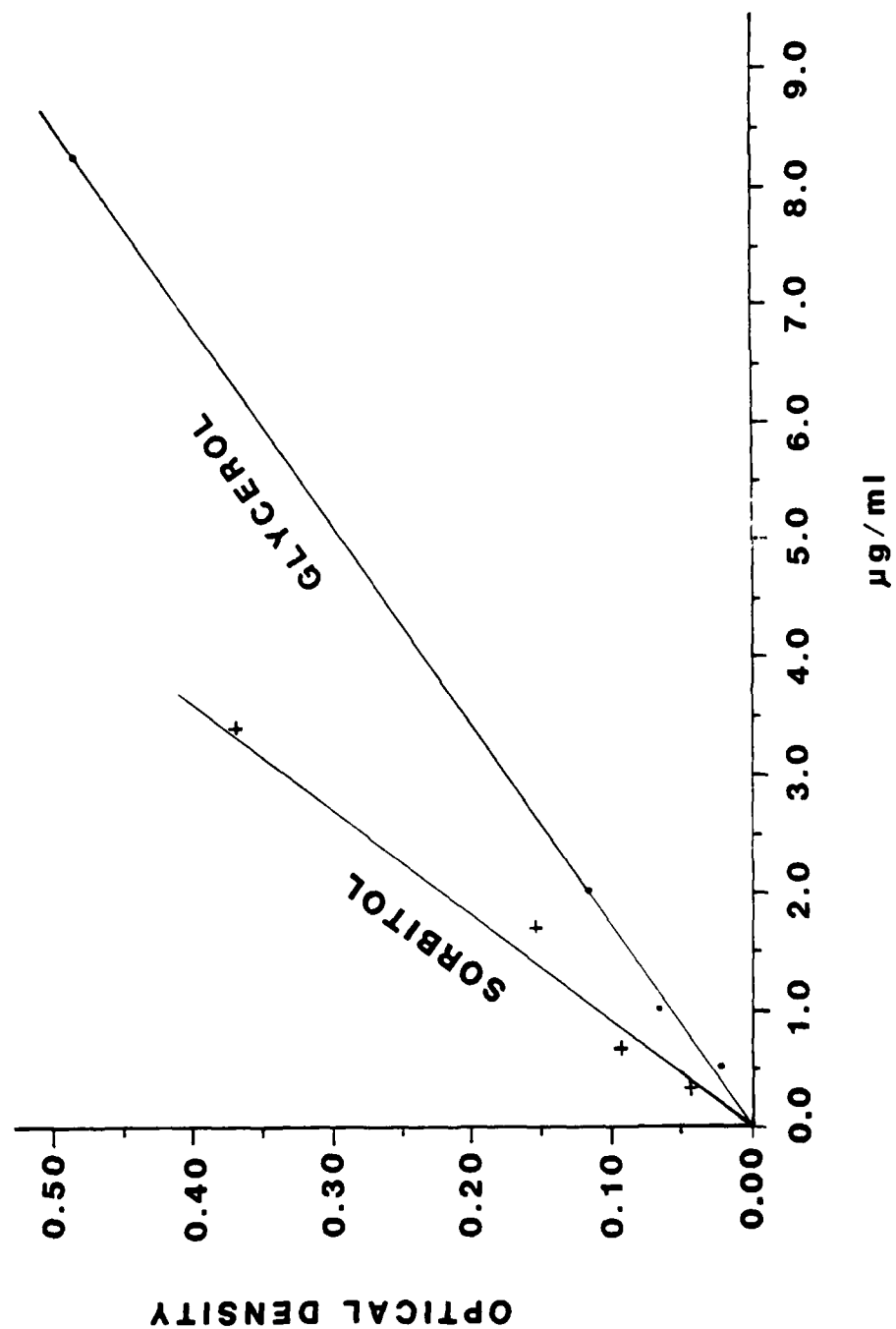
Figure 10. Flow sheet detailing the procedures for the glucose and glycogen assays.

GLUCOSE AND GLYCOGEN ASSAYS

separate and weigh fleas by sex
homogenize in
tissue grinder in:
0.50 ml 80% methanol
rinse twice with 0.75 ml "   "
2.00 ml

spin at 4,000 rpm/5 min

Glucose   Trehalose   Glycogen
(see Fig. 11)

pipette 0.3 ml of   pour off remaining
supernatant into   supernatant
each tube (3)

add 0.9 ml Sigma reagent   add 0.1 ml cold
(1.2 ml in assay tube)   0.6N perchloric acid to
pellet in centrifuge tube

incubate at 37 C for   resuspend pellet by
30 min (with blank & stds*)   stirring with a glass rod

read at 450 nm   let stand in ice
within 30 min   bath for 10 min

add 0.05 ml 1M $KHCO_3$ &
1.0 ml amyloglucosidase

*blank   incubate 60 min at
    0.3 ml 80% methanol   40 C with careful
    0.9 ml Sigma reagent   stirring every 3 min

*standard (3 tubes)
    0.3 ml 5 µg/ml glucose   spin at 4,000 rpm for 5 min
    0.9 ml Sigma reagent

if much   if little
glycogen   glycogen

0.1 ml supernatant   0.3 ml sup.
1.1 ml Sigma reagent   0.9 ml Sigma
reagent

incubate at 37 C for
30 min (with blank & stds*)

read at 450 nm

o-dianisidine as described in Sigma's technical bulletin No. 510. The reactions are as follows:

$$\text{glucose} + 2H_2O + O_2 \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + 2H_2O_2 \quad (1)$$

$$H_2O_2 + \underset{\text{(colorless)}}{\text{o-dianisidine}} \xrightarrow{\text{peroxidase}} \underset{\text{(brown)}}{\text{oxidized o-dianisidine}} \quad (2)$$
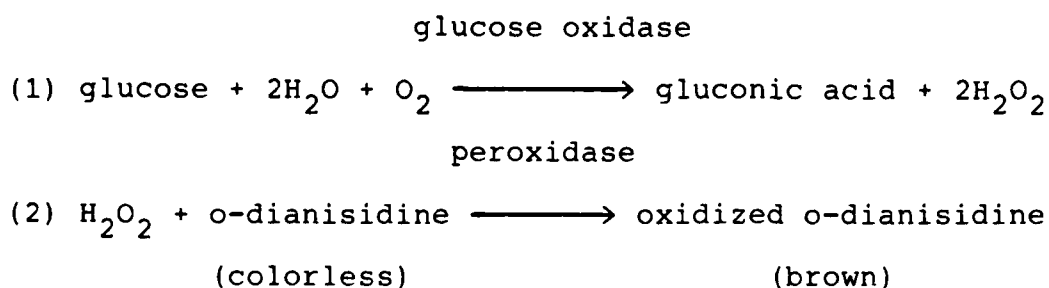
The intensity of the brown color is proportional to the amount of glucose.

The O.D. readings were used to calculate the concentration of glucose, where X = ug glucose/ml in the assay tube.

$$\frac{\text{O.D. standard}}{\text{Conc. standard}} = \frac{\text{O.D. sample}}{X}$$

$$(= 1.25 \text{ } \mu g/ml)$$

The concentration expressed as X was converted to $\mu mol/g$ wet wt. of flea through a series of steps (Appendix 5).

Glycogen Assay

The remaining 1.1 ml of supernatant was carefully decanted and saved for trehalose analysis, and the pellet for glycogen analysis. First, 0.1 ml cold perchloric acid was added and the pellet resuspended with a glass stirring rod. The centrifuge tubes were placed in a ice water bath for 10 minutes, after which 0.05 ml potassium hydroxide and 1.0 ml amyloglucosidase were added. The preparation and dilution of this enzyme are given in Appendix 6. Tubes were incubated in a water bath at 40 C for 60 minutes to hydrolyze the glycogen (Keppler and Decker 1974). Not all glycogen is of

the same molecular weight, therefore reliable quantification requires such hydrolysis be reported as glucose molecules. Tubes were then centrifuged at 4000 rpm for 5 minutes. Three sample tubes for each sex received 0.1 or 0.3 ml supernatant (depending on the amount of glycogen present) and 1.1 or 0.9 ml Sigma reagent, thus yielding 1.2 ml total volume in all tubes. The blank and standard tubes were prepared identically to those for the glucose assay. All tubes were incubated at 37 C for 30 minutes and read at 450nm. Glycogen was calculated and expressed as μmol of glucose/g flea.

Trehalose Assay (Figure 11)

Trehalose analyses were run in conjunction with glucose assays during the second year of the study. Since trehalose has high stability in both alkali and acids, its levels were determined by an anthrone/sulfuric acid method (Wyatt and Kalf 1957; Tanno 1970). Each run consisted of two blanks, three tubes of standard and three sample tubes for each sex. After the final 100 C incubation the tubes were allowed to cool to room temperature and then read at 630 nm. The mean O.D. for the blanks was subtracted from the mean O.D.s for the standard and sample tubes (see appendix 2). These values were used to calculate trehalose concentrations in μmol/g as described for glucose.

Glycerol Assay (Figure 12)

Five to 15 fleas of each sex were homogenized by the same procedure described for glucose/glycogen/trehalose,

Figure 11. Flow sheet detailing procedures for trehalose assay.

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TREHALOSE ASSAY

↓

pipette 0.2 ml of
supernatant into each tube (3)
(blank & standards *)

↓

add 0.2 ml 0.1N H_2SO_4
&
heat at 100 C for 10 min

↓

add 0.15 ml 6N NaOH
&
heat at 100 C for 10 min

↓

chill (tap water bath)
for 15-30 seconds

↓

add 2.0 ml anthrone
&
heat at 100 C for 15 min

↓

allow to cool to room
temperature

↓

read at 630 nm

---
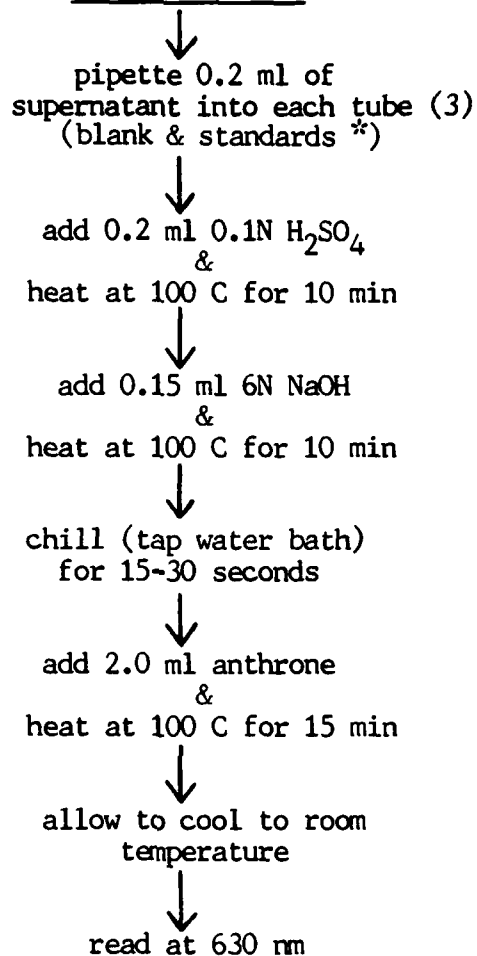
*blank (2 tubes)
  0.2 ml 80% methanol

*standard (3 tubes)
  0.2 ml 50 µg/ml trehalose

Figure 12. Flow sheet detailing procedures for glycerol and sorbitol assays.

GLYCEROL AND SORBITOL ASSAYS

separate by sex and weigh fleas
homogenize in tissue grinder in
0.5 ml deionized/distilled water
(D/D)

↓

rinse twice with 0.75 ml D/D water
(2.0 ml in centrifuge tube)

↓

spin at 4800 rpm for 5 min

↙   ↘

GLYCEROL   ### SORBITOL

in each reaction tube*   in each reaction tube*
    0.4 ml Sol. 1   0.24 ml Sol. 1
    0.56 ml H_2O (D/D)   0.08 ml Sol. 2
    (0.80 ml in blank)   0.08 ml Sol. 3
    0.24 ml sample/standard

↓

add 0.004 ml Sol. 2   ┌─────────────────────────┐
                                     │ from here on keep tubes │
↓   │ covered (Sol. 3 is light│
                                     │ sensitive)   │
#1 reading at 340 nm after 7 min   └─────────────────────────┘

                                     0.44 ml H_2O (D/D)
↓
                                     ↓
add 0.004 ml Sol. 3
                                     add 0.36 ml sample/standard
↓
                                     ↓
#2 reading at 340 nm after 20 min
                                     #1 reading at 492 nm after 2 min

                                     ↓

                                     add 0.020 ml Sol. 4

┌──────────────────────────────┐   ↓
│ ⓔⓐ run had 1 blank, 2 standards│
│ 3 reaction tubes - female fleas│   #2 reading at 492 nm after 30 min
│ 3 reaction tubes - male fleas │
└──────────────────────────────┘

───

*Contents of solutions 1-3 for glycerol and 1-4 for sorbitol analyses
are presented in Appendix 7.

except distilled/deionized water was used instead of 80%
methanol. Glycerol levels were determined by a UV method
(Boehringer Mannheim 1983). Since standards read very con-
sistently, only two tubes were used per run. This enzyma-
tic process is based on the following three reactions:

glycerokinase

(1) glycerol + ATP $\longrightarrow$ glycerol-3-phosphate + ADP

pyruvate kinase

(2) ADP + phosphoenolpyruvate $\longrightarrow$ ATP + pyruvate

lactate dehydrogenase

(3) pyruvate + NADH + H^+ $\longrightarrow$ lactate + NAD^+
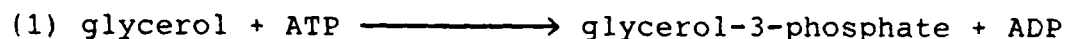
The amount of NADH consumed in reaction #3 is stoichio-
metric with the amount of glycerol in the assay tube. NADH
was determined by its absorption at 340 nm.

The absorbance difference for the blank (reading #1
minus reading #2) was subtracted from the absorbance dif-
ference for each standard and sample tube. Means of the
adjusted readings were used to calculate the glycerol con-
centrations in μmol/g by the same method as outlined for
glucose (Appendix 3).

Sorbitol Assay (Figure 12)

Supernatant from the grind described for glycerol was
also used for sorbitol determinations by the colorimetric
method of Boehringer Mannheim (1983). The following two
enzymatic reactions are the bases for this test:

sorbitol dehydrogenase

(1) D-sorbitol + NAD$^+$ $\longrightarrow$ fructose + NADH + H$^+$

NADH from the above reaction reduces iodonitrotetrazolium

chloride (INT) to formazan in the presence of diaphorase.

diaphorase

(2) NADH + INT + H$^+$ $\longrightarrow$ NAD$^+$ + formazan

The absorbance of formazan is measured at 492 nm against a

distilled water background.

To calculate sorbitol levels the absorbance difference

for the blank (reading #2 minus reading #1) was subtracted

from the absorbance differences of the standard and sample

tubes. The results were averaged and used to calculate

sorbitol concentrations in μmol/g by the same method de-

scribed for glucose (Appendix 4).

Supercooling Points

Supercooling points were taken during the fall, winter

and early spring of 1985-86. A dry ice/acetone bath was

used and the SCPs were measured with a digital thermistor

thermometer (Keithley 866). The thermometer was pretested

and certified accurate by the manufacturer. In addition,

local tests compared against a standard mercury thermometer

in a ice bath showed only a 0.15 C difference in readings.

This accuracy was adequate for SCP determinations. Four

live fleas of the same sex were attached to the thermistor

bead with a thin film of petroleum jelly. This number al-

lowed adequate spacing to prevent inoculative freezing from

one flea to another. The thermistor with attached fleas

and supporting applicator sticks was placed within a series
of four test tubes (Figure 13). Three of these tubes con-
tained cotton wool for insulation purposes which allowed a
cooling rate of 1-2 C per minute. The rate was controlled
by adjusting the depth of the bead and the smallest tube.
A sudden temperature rise of 0.5 to 1.5 C was observed from
the heat of fusion as each flea froze. The temperature at
which that occurred was recorded as the SCP for the entire
insect. Occasional small rises in temperature (0.1 C) were
regarded as evidence for the freezing of individual
appendages.

Statistics

For each mean value in the tables, a standard error of
the mean (S.E.M.) was calculated. In all cases where data
for female and male fleas were pooled, t-tests according to
Sokal and Rohlf (1981) were performed to determine if sex-
ual differences existed. They also were used to compare
means from different assay dates, e.g. mid-March versus mid-
April.

Figure 13. Supercooling apparatus. Fleas were affixed to the thermistor bead with petroleum jelly. Tubes were placed one inside the other and then chilled in a dry ice/acetone bath.

SUPERCOOLING APPARATUS

leads go to a digital thermometer

thermistor taped to applicator sticks

dry ice

acetone
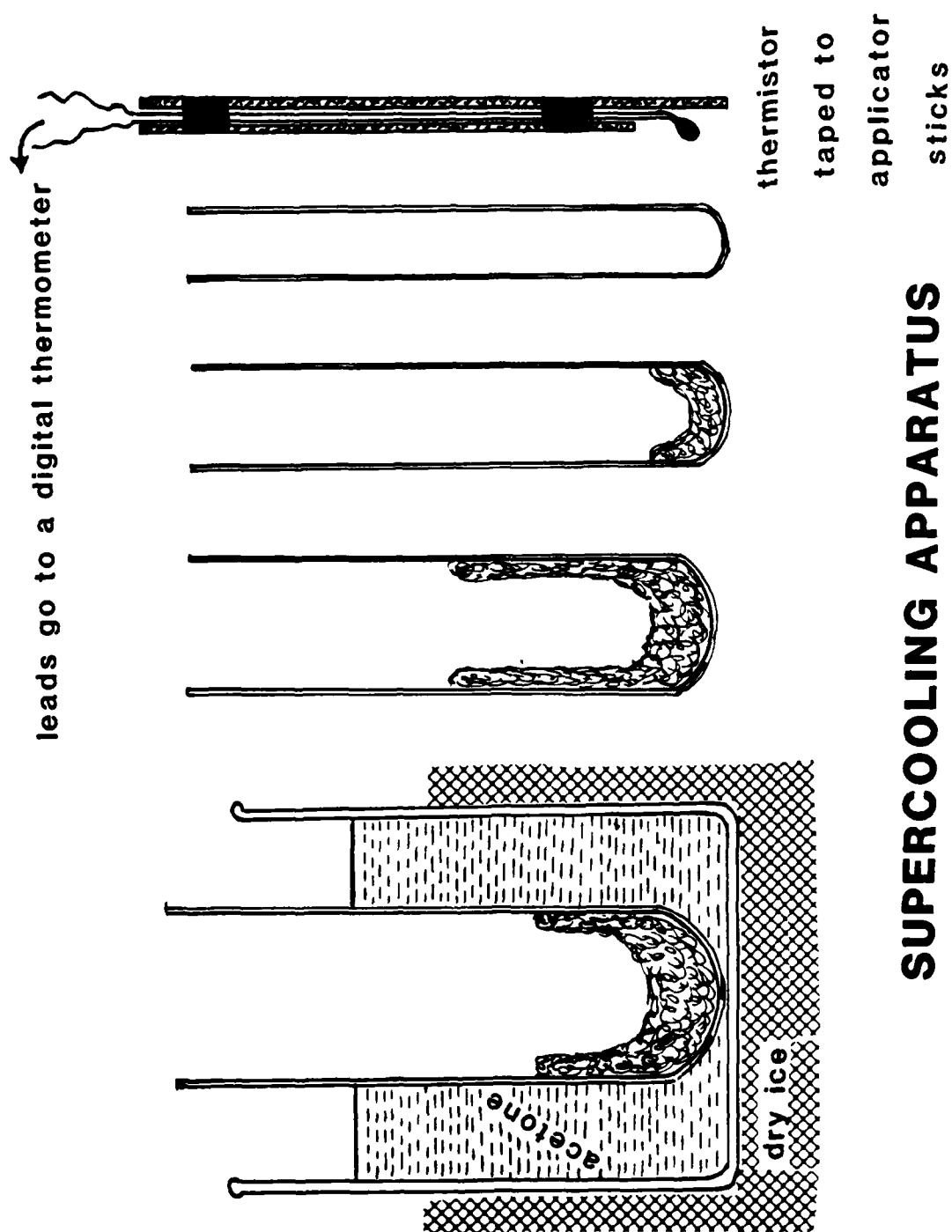
RESULTS

Weight Loss of Fleas

The weight loss experienced by freeze-killed fleas
stored at -25 C is given in Table 1.   Vial 1 showed a 12.7%
decrease after two months and Vial 2 a 12.8% decrease after
four months. This indicates that there was an initial
weight loss after which the weight was stable as long as
the vial remained sealed.   After being opened a second time,
vial 1 showed a 23.9% weight loss (versus time zero) after
four months.   Vial 2 showed a 12.8% loss at the four month
point (initial opening) and a 22.6% loss at six months
after a second opening.   This indicates that fleas lost
additional weight each time the vial was opened, sealed and
returned to the freezer.   After freeze-killing, fleas were
held 1-4 months before being assayed.   Therefore, the
weight losses observed after two (vial 1) and four months
(vial 2) of storage were used to determine an adjustment
factor by which flea weights for each assay were multiplied.
That factor was 1.128.

A second weight loss problem involved living fleas dur-
ing the first half of the cool-down process.   There was a
rapid and substantial decline in the weights of both female
and male fleas during the first 16 days of the cool-down
(Figure 14).   Apparently, a large amount of initial dehy-
dration was followed by a gradual weight loss through mid-
October.   To insure consistency in calculations involving
biomass, two weight lines were extrapolated to time zero.

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Table 1. Weight loss of freeze-killed fleas. Specimens stored in double-sealed containers (glass vials inside a glass jar) at -25 C.

Time	Vial #1 weight of fleas (mg)	Vial #2 weight of fleas (mg)
Zero	13.4	13.3
2 months % change[a]	11.7 (-12.7%)	----
4 months % change[a]	10.2 (-23.9%)	11.6 (-12.8%)
6 months % change[a]	-----	10.3 (-22.6%)

[a] Percent change from time zero weight

Figure 14. Adjusted mean weights of live fleas from the start of the cool-down process through Day 41. Mid-October weights are for freezer-held fleas. Extrapolated line (Broken) used to establish corrected time-zero and 13 C weights.
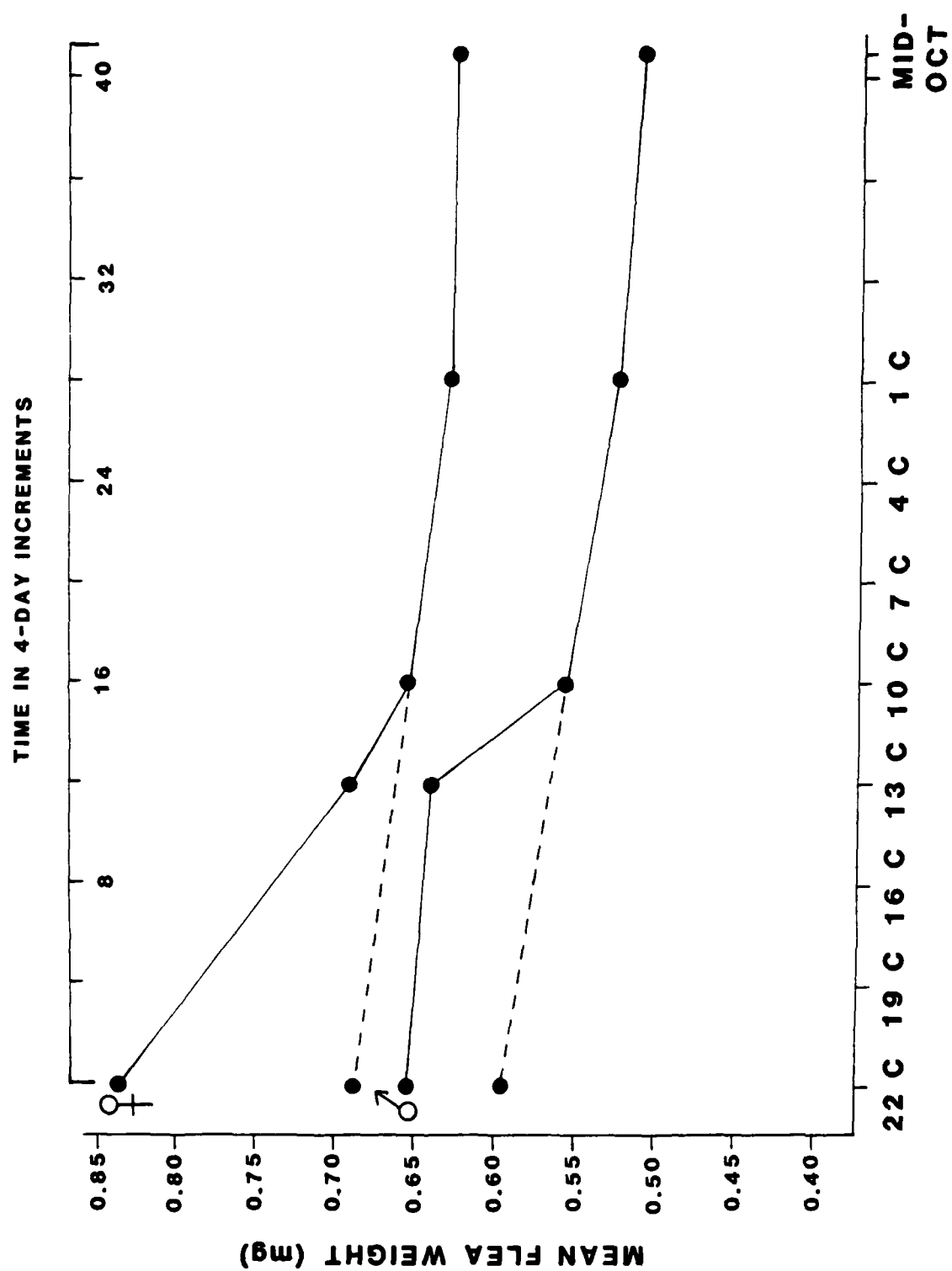
This removed the artifact of hydrated weights in time zero and 13 C fleas. The extrapolated weights were used to calculate the glucose, glycogen, glycerol, sorbitol and trehalose concentrations for these two points.

After placement in a -6 C freezer or on the roof, there were similar but unexplained oscillations in flea weights through mid-February (Figure 15). The freezer-held fleas used for February 1985 glycerol assays were unusually light and probably reflected dehydration from an earlier opening (andperhaps faulty resealing) of the vials. Therefore, glycerol values for freezer-held February 1985 fleas were calculated using the more normal flea weights from the glucose and glycogen assays of the previous week.

Glycerol

Cool-down/Synthesis Induction

Glycerol levels during the entire experimental period are given in Tables 2, 3 and 4. For each glycerol assay in the 1984-85 season, a t-test was performed to determine if there were significant differences between females and males. None were found ($p > 0.05$) between the sexes, therefore, the male and female values were pooled and plotted in Figure 16. Only the cool-down phase is plotted separately. It is obvious that fleas in early September were markedly different in the two years. In 1984, the time zero (22 C) mean glycerol value was 17.5 μmol/g. Twenty days later after having been cooled to, and held at 10 C for four days, the mean glycerol levels had risen over four

Figure 15. Adjusted mean weights of live fleas (1984-1985 and 1985-86) through time. Value for 13 C is from 1985-86 only. F = freezer-held regimen and A = ambient-held regimen. Solid line = females and dashed line = males.

MEAN FLEA WEIGHT (mg)

Table 2. Glycerol levels for freezer-held fleas assayed in 1984-85.
n = number of assays

Date	Glycerol levels in μmol/g for freezer-held female fleas				Glycerol levels in μmol/g for freezer-held male fleas			
	n	Mean	S.E.	Range	n	Mean	S.E.	Range
Time zero	3	19.3	5.9	12.7 - 24.0	2	15.0	4.9	11.5 - 18.4
10 C 4 day	2	61.0	15.5	50.0 - 71.9	2	64.9	1.7	63.7 - 66.1
1 C 4 day	2	182.5	12.2	173.9 - 191.1	2	156.2	25.0	138.5 - 173.9
Mid-October	3	212.2	30.2	184.6 - 244.4	3	215.3	17.4	200.4 - 234.4
Mid-November	3	370.0	35.5	329.1 - 393.0	3	352.1	20.5	348.8 - 374.0
Mid-December	3	455.3	71.4	388.6 - 530.6	3	411.1	23.5	389.5 - 436.1
Mid-January	3	414.8	27.2	383.4 - 431.1	3	414.5	20.7	404.9 - 438.2
Mid-February	2	429.8	3.7	427.1 - 432.4	2	473.5	12.7	464.5 - 482.5
Mid-March	3	462.5	48.5	411.5 - 508.0	2	431.9	26.6	413.1 - 450.7
Mid-April	2	358.0	0.7	357.0 - 358.0	2	389.0	55.7	346.9 - 428.4

Table 3. Glycerol levels for ambient-held fleas assayed in 1984-85.
n = number of assays

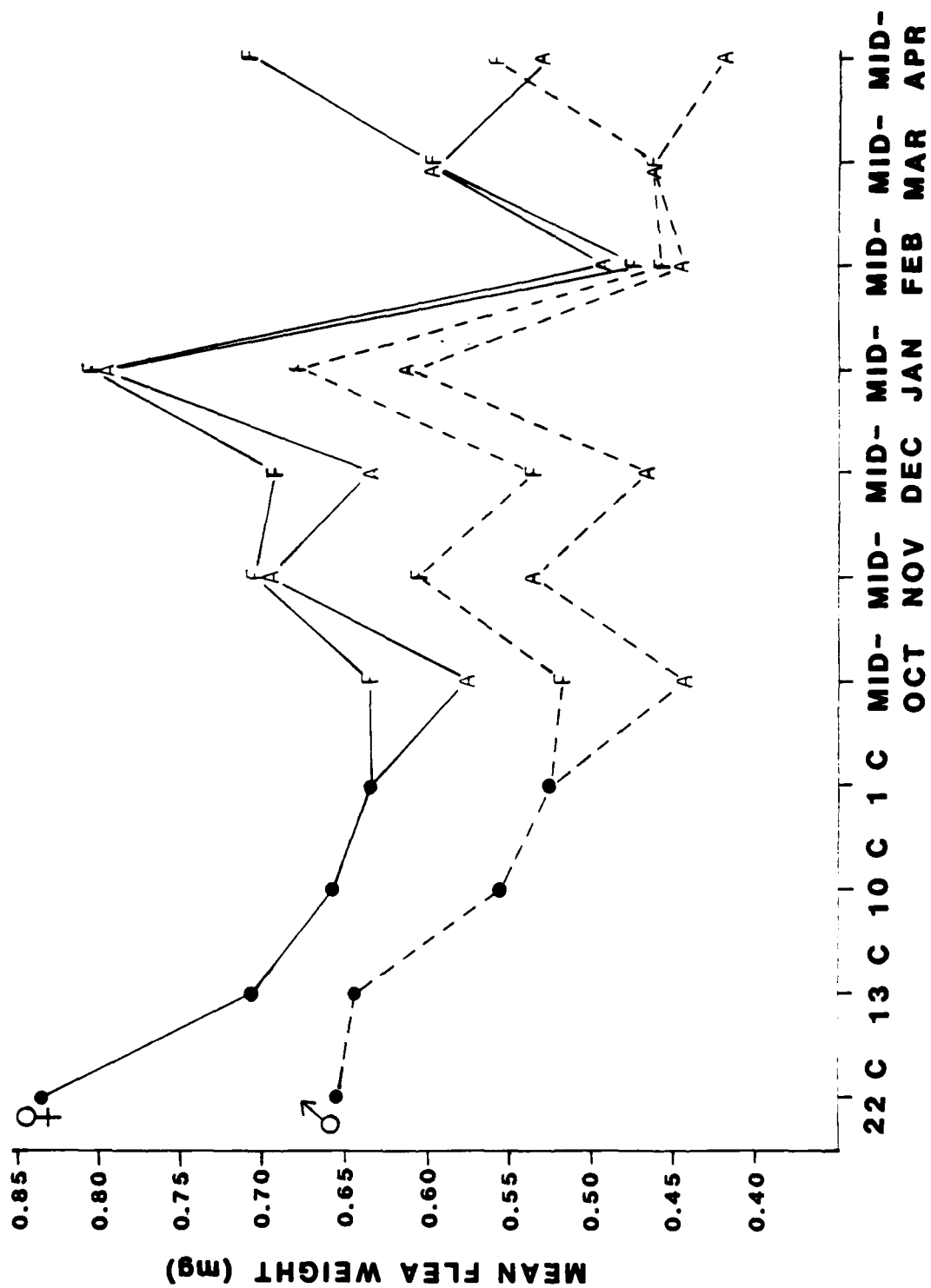| Date	Glycerol levels in µmol/g for ambient-held female fleas				Glycerol levels in µmol/g for ambient-held male fleas			
	n	Mean	S.E.	Range	n	Mean	S.E.	Range
Time zero	3	19.3	5.9	12.7 - 24.0	2	15.0	4.9	11.5 - 18.4
10 C 4 day	2	61.0	15.5	50.0 - 71.9	2	64.9	1.7	63.7 - 66.1
1 C 4 day	2	182.5	12.2	173.9 - 191.1	2	156.2	25.0	138.5 - 173.9
Mid-October	3	84.7	5.1	79.5 - 89.6	3	87.5	15.8	69.9 - 100.5
Mid-November	3	267.5	33.9	228.8 - 291.9	3	232.2	29.4	206.7 - 264.4
Mid-December	3	482.8	46.8	429.8 - 518.3	3	479.2	33.3	456.1 - 517.3
Mid-January	2	387.4	45.4	355.3 - 419.5	3	367.7	26.9	337.4 - 388.5
Mid-February*	3	534.2	23.2	517.8 - 560.8	2	595.0	69.4	546.9 - 645.0
Mid-March*	3	498.9	73.6	419.1 - 564.2	3	469.4	39.3	441.7 - 514.4
Mid-April*	3	246.3	48.0	210.7 - 301.3	3	267.3	6.8	262.3 - 275.2

*From 1985-86 season only.

Table 4. Glycerol levels for freezer-held fleas assayed in 1985-86.
n = number of assays

Date	n	Glycerol levels in μmol/g for ambient-held female fleas			n	Glycerol levels in μmol/g for ambient-held male fleas		
		Mean	S.E.	Range		Mean	S.E.	Range
Time zero	4	97.8	29.6	69.7 - 129.2	3	104.8	34.2	68.3 - 136.1
13 C 4-day	2	91.6	24.7	74.1 - 109.0	2	107.5	16.7	95.7 - 119.2
10 C 4-day	1	113.4	----	--------	2	87.2	3.9	84.4 - 89.4
1 C 4-day	2	147.5	58.9	105.8 - 189.1	1	100.0	----	--------
10 C 22-day	1	236.8	----	--------	2	170.5	25.5	152.5 - 188.5
Mid-October	2	389.6	52.4	352.5 - 426.6	2	396.6	37.1	370.4 - 422.8
Mid-November	2	453.8	7.3	448.6 - 458.9	2	479.9	50.8	443.9 - 515.8
Mid-February	1	656.0	----	--------	1	681.6	----	--------
Mid-March	2	671.7	156.1	561.3 - 782.0	2	694.6	15.7	683.5 - 705.7
Mid-April	2	773.1	26.1	754.6 - 791.5	2	684.6	46.4	651.8 - 717.4

Figure 16. Mean glycerol levels versus time and regimens, 1984-85/ 1985-86. Male and female values pooled for both years except for the cool-down phase.

65
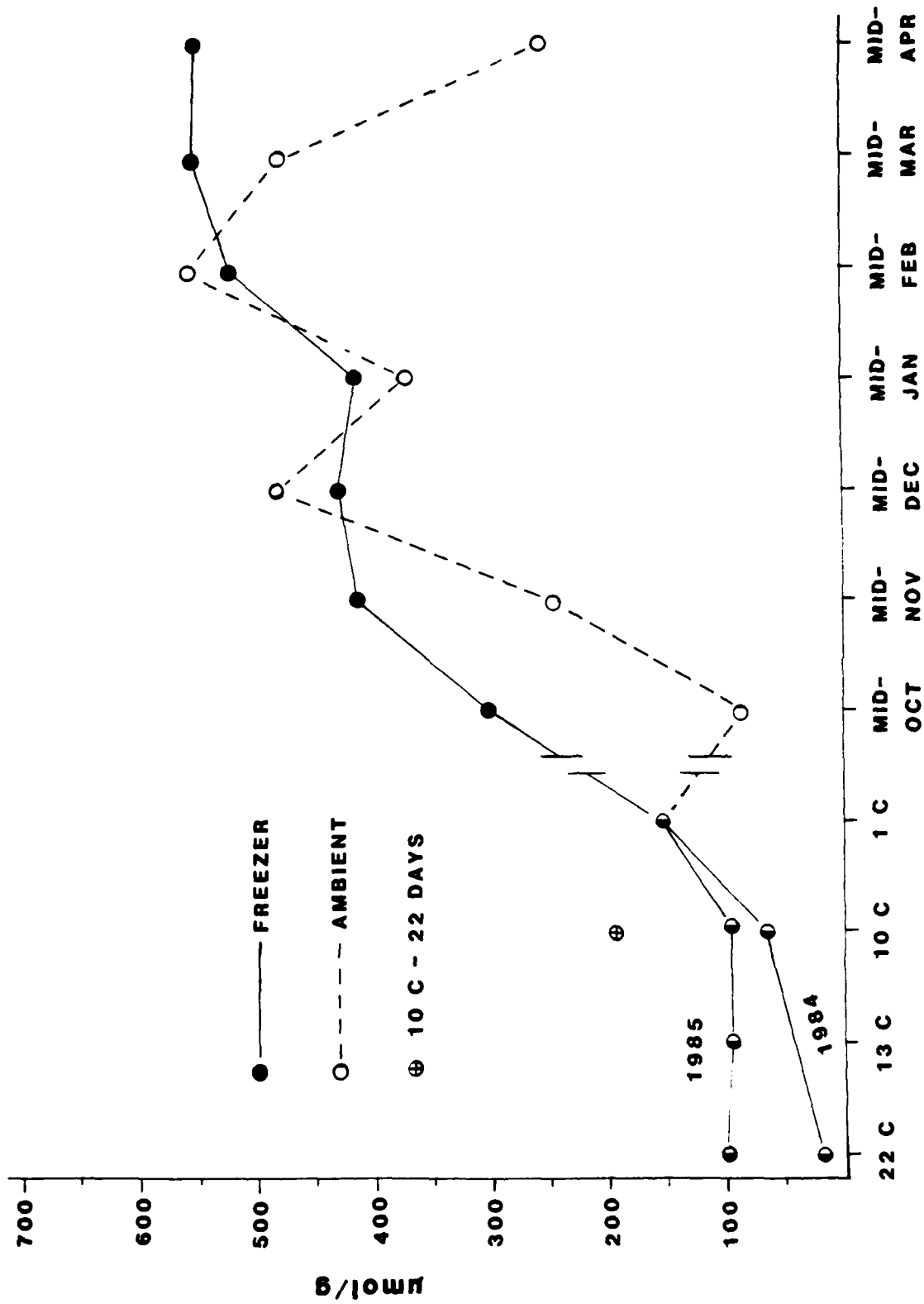
fold. A t-test showed that the difference between the mean values for 22 C and 10 C were significant $(0.01 < P < 0.05)$. This difference indicates that glycerol synthesis had been induced at or prior to 10 C. However, in 1985, the glycerol levels at time zero were 101.3 μmol/g or almost six times higher than the previous year. A comparison of temperatures for August and early September indicates that in 1985 the daily minimum temperature dropped to 10 C or less 10 times (Table 5). In 1984, such coolness was reached only six times.

In 1985, the cool-down to 10 C produced no effect on glycerol levels. However, some fleas were held at 10 C for 22 days to determine if, once induced, glycerol synthesis would continue without further cooling (Table 4 and Figure 16). The glycerol level at this time was 201.4% higher than for the fleas cooled to 10 C for four days.

Freezer-held Regimen

After being moved from 1 C to -6 C, glycerol levels increased steadily through mid-November (Table 2 and Figure 16). The combined means (female + male and 1984-85 + 1985-86 seasons) increased to 285.5 μmol/g by mid-October and to 403.4 μmol/g by mid-November. From mid-February through mid-April, mean glycerol remained relatively constant, but at levels 25% higher than earlier.

Ambient-held Regimen

After being moved from 1 C to ambient roof-top conditions on October 4, 1984, the mean glycerol levels declined

Table 5. Temperatures for Grand Forks, North Dakota, August 14 -
September 5, 1984 and 1985. The temperatures are reported
by the Geography Department, University of North Dakota. The
critical temperature to trigger glycerol synthesis is 10 C.

| | 1984 Temperatures | | 1985 Temperatures | |
Date	Maximum	Minimum	Maximum	Minimum
Aug. 14	28.9	20.6	23.3	8.9
15	26.1	13.9	25.6	8.9
16	30.0	14.4	25.6	10.6
17	28.9	16.1	16.7	13.9
18	30.0	12.8	15.6	10.0
19	30.6	14.4	19.4	5.6
20	22.8	17.8	21.7	5.0
21	25.6	12.8	25.0	11.1
22	21.1	11.1	29.4	13.9
23	25.0	7.8	22.8	13.9
24	25.6	15.0	23.9	14.4
25	30.6	16.1	26.1	8.3
26	34.4	18.9	27.2	13.9
27	35.6	16.1	22.8	8.3
28	35.6	15.0	15.6	7.2
29	25.0	7.8	23.3	11.1
30	23.3	10.6	23.3	11.1
31	20.6	7.8	25.6	16.7
Sep. 1	16.7	11.1	22.2	9.4
2	20.6	10.6	17.8	13.9
3	22.8	6.7	21.1	18.3
4	21.1	6.1	22.8	13.9
5	22.8	3.9	26.1	10.0

49% during an 11 day period (Table 3 and Figure 16). This was opposite to that observed in freezer-held fleas. Average daily temperatures for most of this time were above normal and above 10 C (Figure 17). From mid-October to mid-November with temperatures consistently below 10 C, glycerol levels increased 290% Although monthly oscillations were substantial, December through March values were similar to those occurring in freezer-held fleas. These four months include the coldest temperatures for a North Dakota winter. A significant decrease (t-test, $p < .001$) in mean glycerol levels was observed between mid-March and mid-April. This decline coincides with the warming temperatures of spring.

Sorbitol

Sorbitol levels (Table 6) were low at time zero with a mean value of 1.2 μmol/g (combined female and male). The levels remained low and were barely detectable during the cool-down process and through the last assays run in mid-February. By that time it was apparent that sorbitol levels were low and unchanging.

Trehalose

Trehalose assays were performed only during the 1985-86 season (Table 7). Although there were fluctuations during the cool-down period, mean values remained low in both freezer-held and ambient-held fleas (Figure 18). The two-fold increase at 13 C followed by a rapid decline at 10 C may be a fringe effect of the concurrent but larger event of glycerol/glycogen metabolism. Similar oscillations in

Figure 17. Temperatures for 1984-85. Curve is drawn using mean temperatures for five day periods. Vertical lines connect maximum and minimum temperatures for each five day increment. Asterisk indicates warm period after 1 C cooled fleas were placed on the roof.
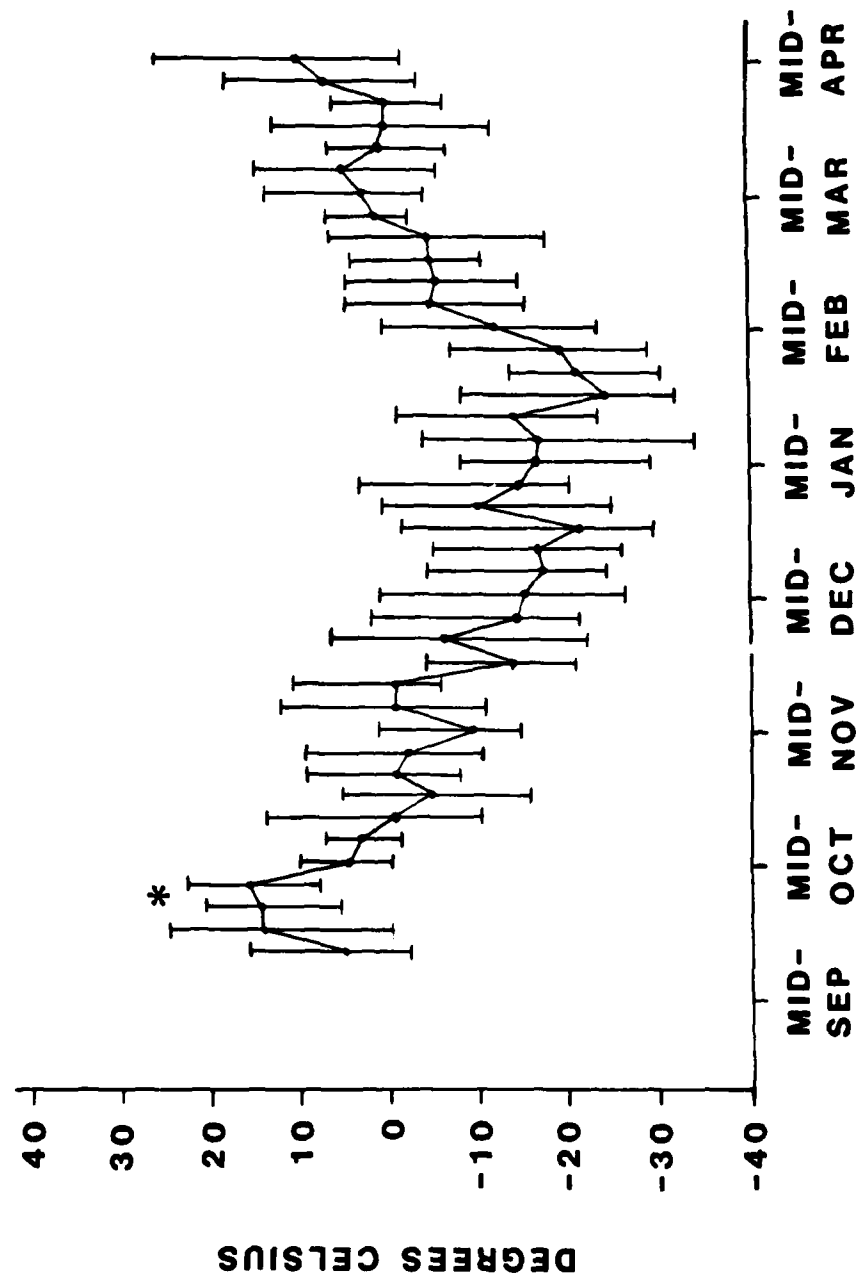
Table 6. Sorbitol levels for 1984-85. All fleas were subjected to the same cool-down process; therefore, time-zero through 1 C are listed only once. n = number of assays

Date	Sorbitol levels in μmol/g for female freezer-held fleas			Sorbitol levels in μmol/g for male freezer-held fleas			Sorbitol levels in μmol/g for female ambient-held fleas			Sorbitol levels in μmol/g for male ambient-held fleas		
	n	Mean	Range	n	Mean	Range	n	Mean	Range	n	Mean	Range
Time-zero	3	1.2	0.9 – 1.5	3	1.2	0.8 – 1.6						
10 C 4-day	3	0.7	0.3 – 1.2	3	1.3	1.3 – 1.4						
1 C 4-day	3	1.2	0.7 – 1.5	3	1.2	0.6 – 1.4						
Mid-October	2	1.1	0.6 – 1.5	2	2.2	1.8 – 2.6	2	1.1	0.6 – 1.6	2	2.2	1.9 – 2.5
Mid-November	2	1.5	1.5 – 1.5	2	2.3	1.8 – 2.7	1	0.8	------	1	0.8	------
Mid-December	2	1.3	0.2 – 2.3	2	3.1	3.1 – 3.1	2	1.3	1.0 – 1.6	2	1.5	0.6 – 2.5
Mid-January	1	0.9	------	1	0.3	------	2	1.4	0.4 – 2.3	1	3.2	------
Mid-February	1	0.6	------	1	0.9	------	-	--	------	-	--	------

Table 7. Trehalose levels for 1985-86. Date column: F = Freezer, A = Ambient, n = number of assays

Date [a]	Trehalose levels in μmol/g for female fleas				Trehalose levels in μmol/g for male fleas			
	n	Mean	S.E.	Range	n	Mean	S.E.	Range
Time-zero	3	18.5	2.2	16.0 - 20.1	3	16.0	1.5	14.6 - 17.5
13 C 4-day	3	32.3	3.0	30.5 - 35.8	2	37.8	7.6	32.4 - 43.2
10 C 4-day	2	13.7	1.3	12.7 - 14.6	2	11.1	1.3	10.1 - 12.0
1 C 4-day	3	16.3	1.3	14.9 - 17.2	3	16.7	0.2	16.5 - 16.9
Mid-October-F	3	26.5	8.1	19.9 - 35.6	2	27.9	1.0	27.2 - 28.6
Mid-November-F	1	20.6	--	--------	1	18.3	--	--------
Mid-February-A	1	13.8	--	--------	2	8.4	8.5	2.4 - 14.4
Mid-March-A	4	30.1	7.1	20.5 - 37.5	2	31.4	16.6	19.6 - 43.1
Mid-April-A	2	15.0	8.8	8.8 - 21.2	2	20.5	7.7	15.0 - 25.9

[a] No analyses made in December or January

Figure 18. Mean trehalose levels versus time and regimen for 1985-
86. Male and female values pooled.
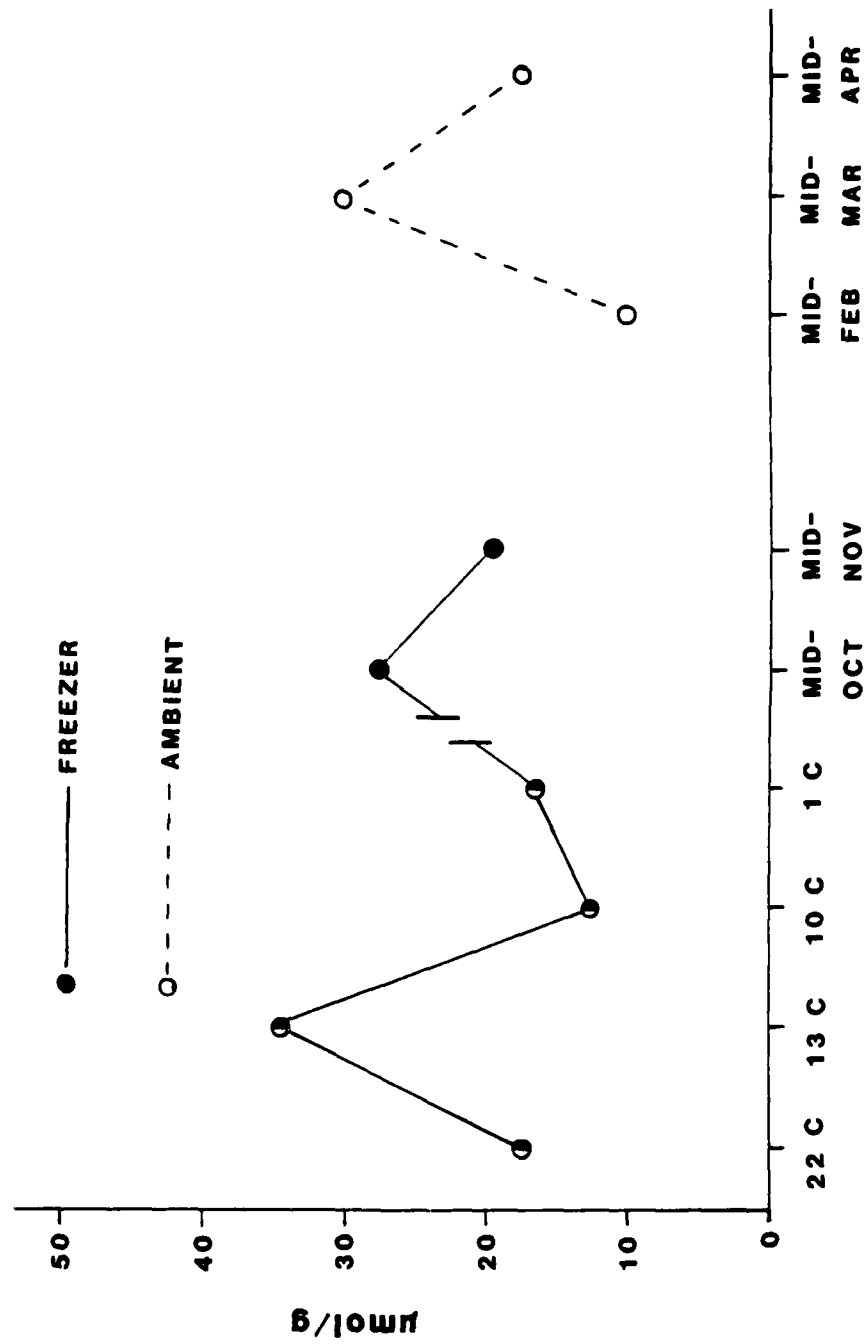
ambient-held fleas in the spring may reflect the seasonal reversal of the system.

Glucose

Mean glucose levels tended to remain low, usually ranging from 10 to 20 μmol/g (Table 8 and Figure 19). There was a transitory 3-4 fold rise in mid-October glucose levels when the rate of glycogen degradation was high. The decline in glucose from the mid-October peak was more gradual for ambient than for the freezer-held fleas.

Glycogen

In both years, mean glycogen levels (Tables 9 and 10 and Figure 20) expressed in glucose units were initially high (359.5 μmol/g). From time-zero to the 13 C and 10 C points, the levels decreased and then a slight increase occurred between 10 C and the 1 C point. This increase was not statistically significant. During accelerated glycerol synthesis in mid-October through mid-November, glycogen levels decreased at a rapid rate (Figure 21). The high levels of glycerol attained during the winter corresponded with low glycogen levels in both freezer- and ambient-held fleas.

In ambient-held fleas, the mid-April values for glycogen showed a 39% increase concomitant with the decreased glycerol levels. Such changes were not observed in freezer-held fleas.

Supercooling points

During 1985-86, supercooling points (Table 11 and Figure 22) were determined at various times to ascertain: (1) if

Table 8. Glucose levels for freezer-held and ambient-held fleas. All fleas were subjected to the same cool-down process; therefore, time-zero through 1 C are listed only once. n = number of assays

Date	Glucose levels in μmol/g for freezer-held female fleas			Glucose levels in μmol/g for freezer-held male fleas			Glucose levels in μmol/g for ambient-held female fleas			Glucose levels in μmol/g for ambient-held male fleas		
	n	Mean	S.E.	n	Mean	S.E.	n	Mean	S.E.	n	Mean	S.E.
Time-zero*	6	17.1	5.2	5	14.9	5.6						
13 C 4-day*	3	14.7	10.8	3	12.9	11.1						
10 C 4-day*	8	15.9	4.1	5	16.5	4.4						
1 C 4-day*	5	11.3	4.2	4	13.2	4.9						
Mid-October	3	30.2	3.4	3	29.9	5.8	3	38.3	8.8	3	39.5	3.6
Mid-November	3	12.2	1.6	3	10.1	1.0	3	21.7	4.2	3	34.7	3.7
Mid-December	3	14.1	2.9	3	15.4	6.0	2	12.1	0.2	2	20.1	6.5
Mid-January	3	13.1	1.8	3	15.6	4.1	3	14.5	2.9	3	16.5	1.1
Mid-February*	2	21.2	3.6	2	18.9	3.2	3	15.9	3.3	3	13.9	2.8
Mid-March*	3	14.0	2.3	3	22.2	7.7	3	10.7	1.4	2	12.6	2.4
Mid-April*	3	15.9	0.8	3	14.1	4.3	3	8.4	4.2	3	10.9	4.7

*Combined values from 1984-85 and 1985-86 seasons; all other values from 1984-85 season

Figure 19. Mean glucose levels versus time and regimen for 1984-85, 1985-86. Male and female values pooled.
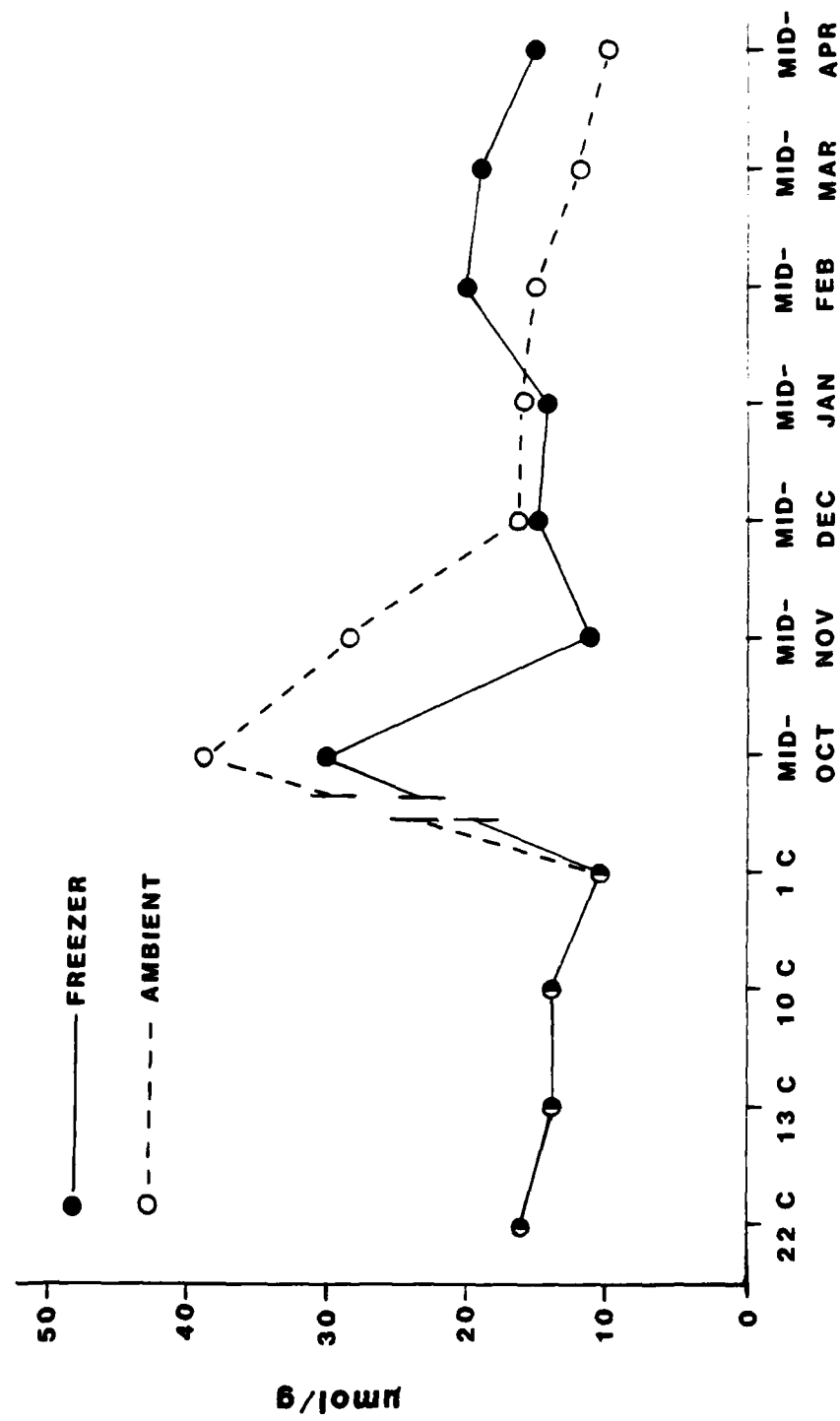
Table 9. Glycogen levels in glucose units for freezer-held fleas. n = number of assays

Date	Glycogen levels as µmol glucose/g wet wt. of freezer-held female fleas				Glycogen levels as µmol glucose/g wet wt. of freezer-held male fleas			
	n	Mean	S.E.	Range	n	Mean	S.E.	Range
Time-zero*	10	368.0	61.0	276.0 - 446.5	10	351.0	79.4	252.6 - 471.6
13 C 4-day*	3	391.5	44.6	386.1 - 438.6	3	322.5	23.2	296.3 - 340.5
10 C 4-day*	7	325.0	77.4	212.1 - 439.4	6	309.4	84.0	212.8 - 391.7
1 C 4-day*	5	328.6	49.6	243.6 - 361.5	3	317.0	21.4	295.9 - 338.3
Mid-October	3	224.5	63.0	151.7 - 261.1	3	159.2	1.0	158.0 - 159.8
Mid-November	3	89.0	5.3	83.2 - 93.5	3	74.8	41.8	45.9 - 122.8
Mid-December	3	69.1	11.1	57.0 - 78.7	3	92.0	35.7	50.8 - 114.2
Mid-January	3	25.1	4.5	22.3 - 30.3	3	19.0	4.4	14.0 - 22.2
Mid-February	3	16.7	2.5	13.8 - 18.5	3	17.1	2.7	14.0 - 18.8
Mid-March	3	13.9	5.5	9.4 - 20.0	3	19.1	6.6	12.8 - 25.9
Mid-April	3	15.1	6.3	10.7 - 22.3	3	13.9	3.0	10.5 - 15.9

*Combined values from 1984-85 and 1985-86 seasons; all other values from 1984-85 season.

Table 10. Glycogen levels in glucose units for ambient-held fleas. n = number of assays

Date	Glycogen levels as μmol glucose/g wet wt. of ambient-held female fleas				Glycogen levels as μmol glucose/g wet wt. of ambient-held male fleas			
	n	Mean	S.E.	Range	n	Mean	S.E.	Range
Time-zero*	10	368.0	61.0	276.0 - 446.5	10	351.0	79.4	252.6 - 471.6
13 C 4-day*	3	391.5	44.6	386.1 - 438.6	3	322.5	23.2	296.3 - 340.5
10 C 4-day*	7	325.0	77.4	212.1 - 439.4	6	309.4	84.0	212.8 - 391.7
1 C 4-day*	5	328.6	49.6	243.6 - 361.5	3	317.0	21.4	295.9 - 338.3
Mid-October	3	257.5	30.9	238.1 - 293.1	2	252.0	4.3	248.9 - 255.0
Mid-November	3	77.5	6.8	70.3 - 83.9	3	86.2	2.9	83.5 - 89.3
Mid-December	3	18.5	4.5	15.1 - 23.6	3	21.4	3.3	17.7 - 24.0
Mid-January	3	17.9	5.8	12.4 - 24.0	3	15.0	3.4	11.9 - 18.7
Mid-February*	2	17.5	5.3	13.8 - 21.3	3	22.2	2.0	20.5 - 24.4
Mid-March*	4	36.1	5.6	27.8 - 39.0	2	29.7	13.0	20.5 - 38.9
Mid-April*	3	52.5	45.1	25.7 - 104.7	3	41.7	5.8	37.3 - 48.3

*Combined values from 1984-85 and 1985-86 seasons; all other values from 1984-85 season.

Figure 20. Mean glycogen levels expressed as μmol glucose/g wet
wt. of flea versus time and regimen. Male and female values
pooled. Time-zero through 1 C and February through April ambient
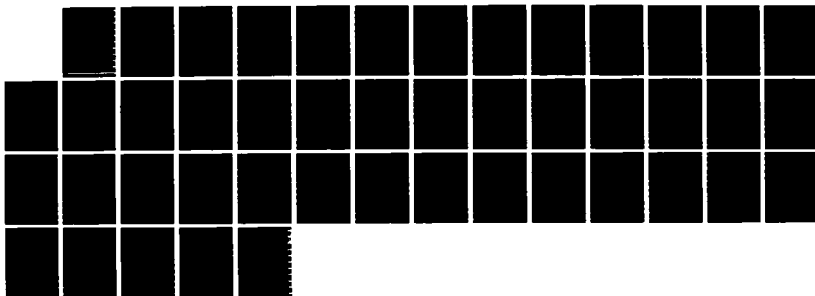values are combined from both years; all others are from 1984-85
only.
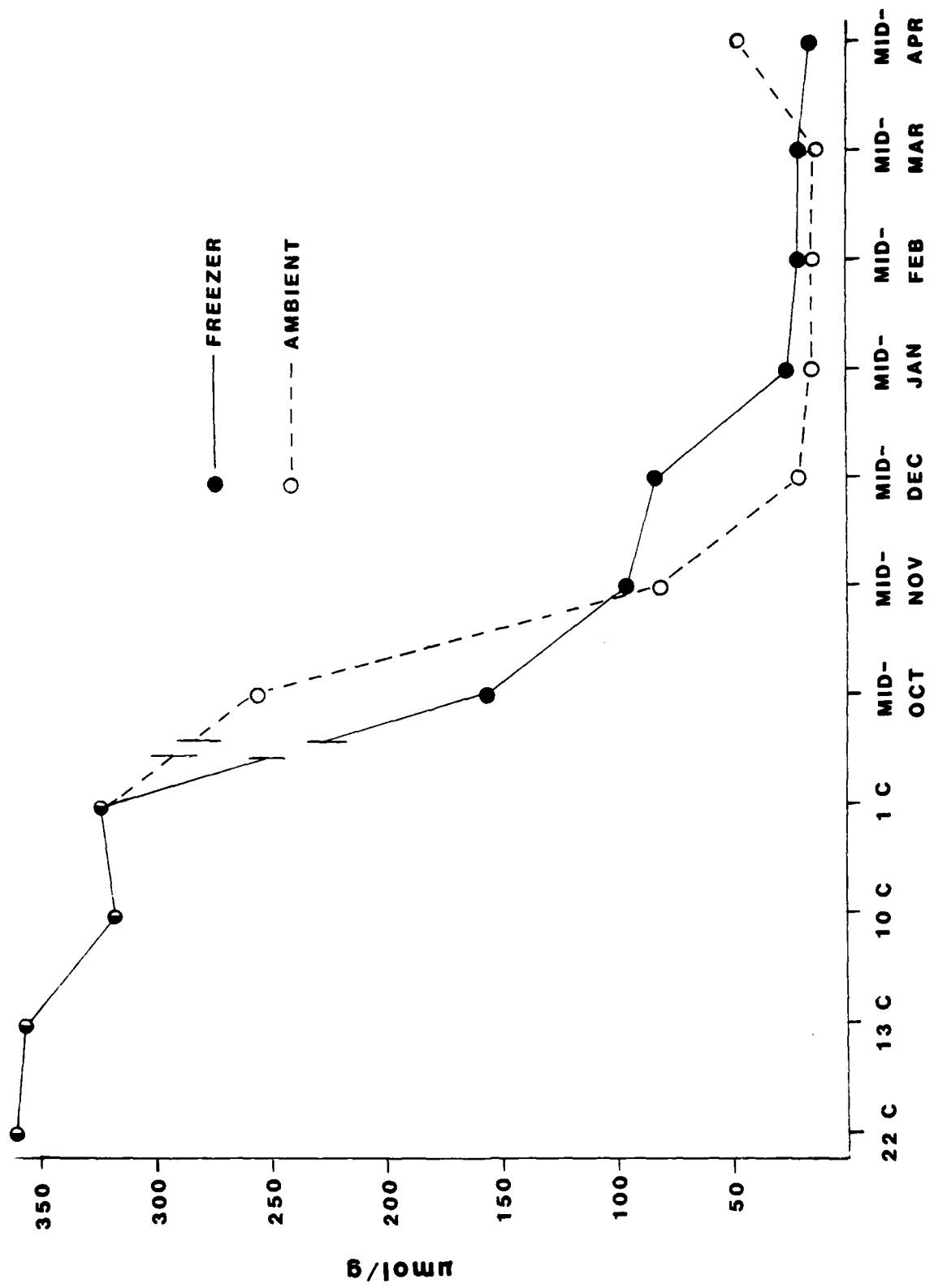
MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDA: DS-1963-A

Figure 21. Comparison of glycerol and glycogen values from time
zero through mid-April. Parameters as described for Figures 16 and
20.
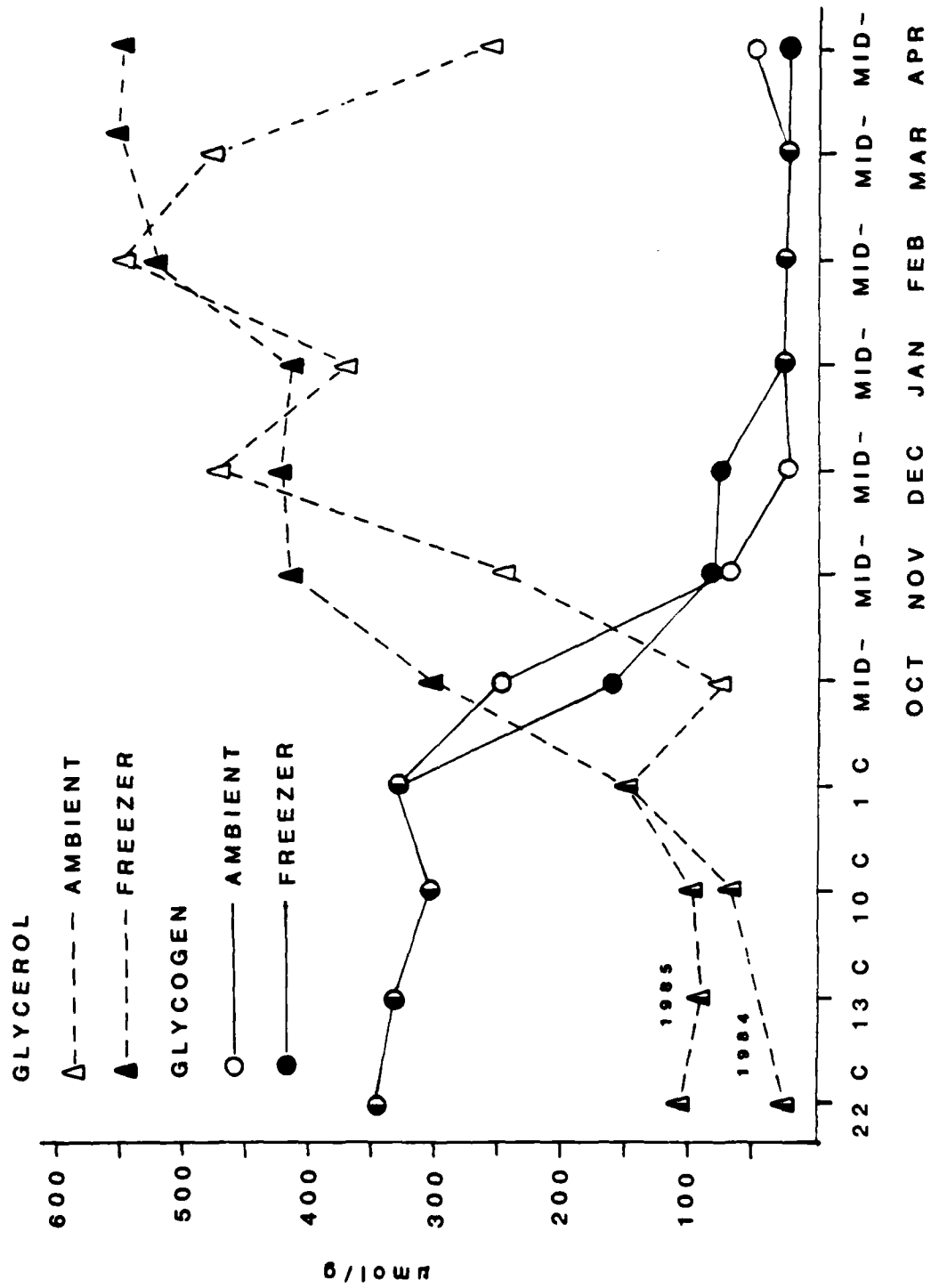
Table 11. Supercooling points for adult Ceratophyllus idius, 1985-86.
In the date column F = freezer-held and A = ambient-held. n = number of individual fleas

Date		Supercooling points for females in degrees Celsius				Supercooling points for males in degrees Celsius		
	n	Mean	S.E.	Range	n	Mean	S.E.	Range
Time-zero	10	-19.7	0.9	-18.3 - -21.0	10	-19.0	2.5	-16.1 - -22.0
10 C 4 days	8	-25.5	1.4	-23.5 - -27.2	7	-24.1	1.7	-21.8 - -26.1
1 C 4 days	8	-26.1	1.9	-23.4 - -29.0	8	-27.5	2.0	-24.5 - -29.8
10 C 22 days	8	-27.1	1.4	-24.8 - -28.5	8	-26.7	1.7	-23.8 - -29.0
Mid-October-F	8	-28.1	1.1	-26.4 - -29.5	8	-27.5	1.4	-26.1 - -30.4
Mid-November-F	7	-30.0	1.1	-28.7 - -31.3	7	-29.0	1.5	-27.5 - -31.1
Mid-November-A	8	-29.9	1.0	-28.1 - -31.4	8	-29.0	1.3	-27.4 - -30.4
Mid-February-F	8	-29.8	2.1	-26.5 - -31.4	8	-29.9	1.2	-28.7 - -31.4
Mid-February-A	8	-29.7	2.0	-27.5 - -32.7	7	-29.8	1.8	-26.7 - -32.0
Mid-March-F	7	-29.3	2.4	-26.1 - -32.0	7	-30.5	1.6	-28.4 - -31.7
Mid-March-A	7	-27.4	1.3	-26.4 - -29.9	7	-28.7	1.4	-26.6 - -30.8
Mid-April-F	7	-29.7	2.1	-25.8 - -31.6	6	-30.2	2.1	-26.9 - -32.1
Mid-April-A	8	-26.3	1.2	-24.7 - -28.1	8	-27.8	1.1	-26.1 - -29.4

All determinations were done using live fleas on the same days that other fleas from the same vials were freeze-killed for assays.

Figure 22. Average supercooling points for ambient- and freezer-
held fleas, 1985-86. Male and female values pooled. Vertical
lines = one standard error.
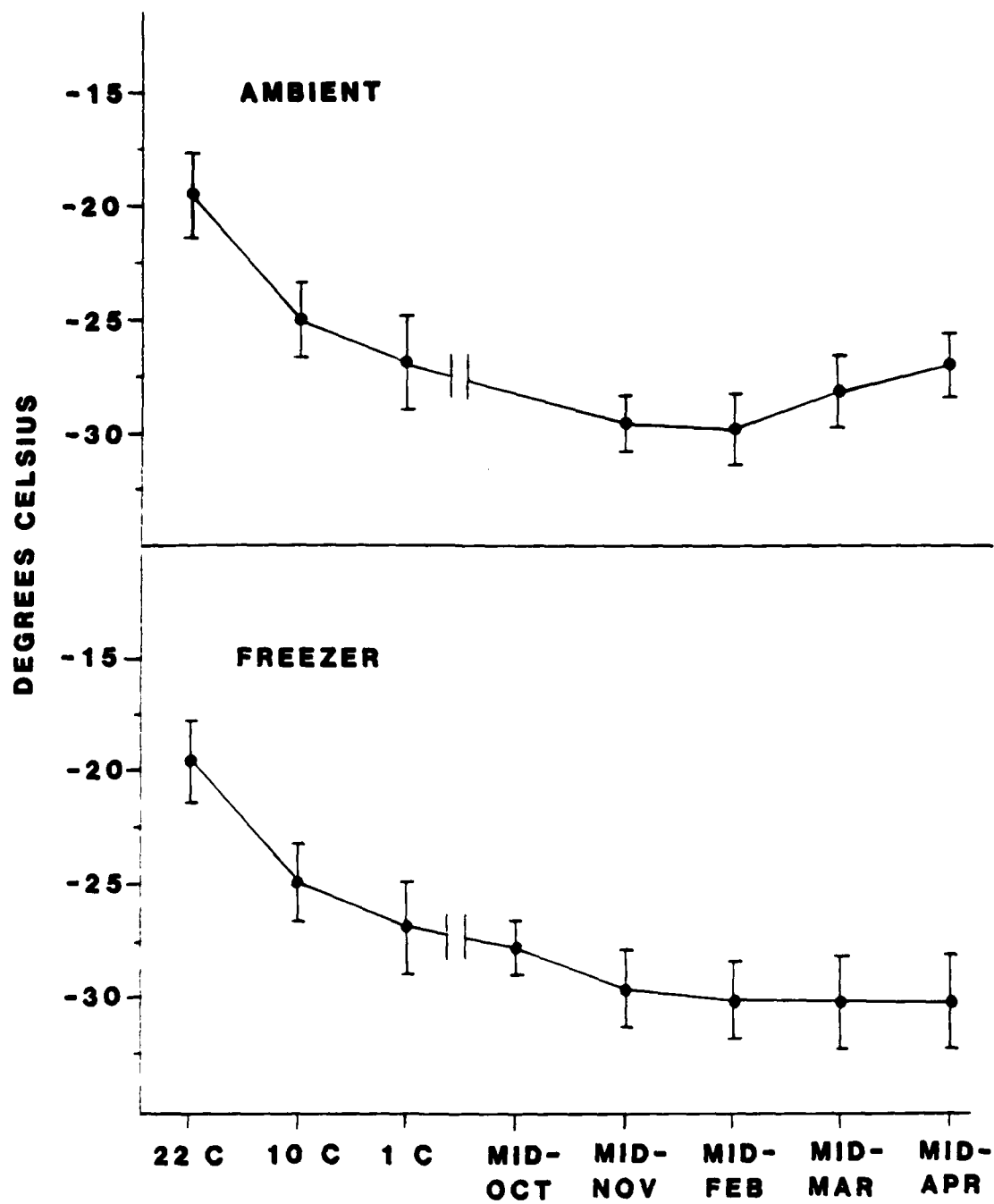
the fleas were freeze-tolerant (with high SCPs) or freeze-susceptible (with low SCPs), and (2) if freeze-susceptible, how much was the supercooling capacity of the fleas increased. The time-zero values were determined on fleas held at 22 C for about three weeks, and they had relatively low SCPs (-19.7 C for females and -19.0 C for males).

During cool-down to mid-November (ambient and freezer), there was a slow, steady lowering of SCPs until they stabized in the -29 to -30 C range. They remained at that level through mid-March in the freezer, but showed a rise in the mid-March ambient readings. This followed the same general pattern seen in glycerol levels. In fleas held for 22 days at 10 C, the SCPs became lower as glycerol levels increased over the same time period.

Simulated Mid-winter Thaw

Mid-winter thaws are common in North Dakota, but the effects these have on the cold-hardiness of fleas is unknown. Temperatures needed to simulate a mid-winter thaw were obtained from weather data for January 4-8, 1985. Over that five day period the daily high temperatures averaged 2.2 C. At mid-January 1985, living fleas for this experiment were moved from -6 C to 2 C for seven days. The glycerol levels after seven days (366.5 μmol/g) were significantly lower (p < 0.01, t-test) than mid-January freezer glycerol levels (414.0 μmol/g).

Reversibility/Temperature Dependence of Glycerol Synthesis

To test the hypothesis that the synthesis of glycerol is

temperature dependent and reversible, 20 vials were moved from -6 C to 15 C on the same day that the mid-October freezer fleas were freeze-killed. Data for this experiment are presented in Tables 12, 13, 14, 15 and 16 and Figure 23. Two days after transfer to 15 C, mean glycerol levels had decreased 25.9% and by day four, they were down 40.7%. The experiment was originally designed to run four days before recooling the survivors. There was a surplus of female fleas, and these were left at 15 C for eight days. The glycerol values for these showed a continued, steady decrease to 29.8% of levels present at day zero. All other surviving 4-day warmed fleas were returned to -6 C for 18 days. Analysis of these fleas showed that glycerol synthesis was resumed with levels returning to 76.2% of prewarmed values. As with earlier but slower temperature changes, glycogen levels again corresponded with altered glycerol levels.

Changes in the SCPs closely followed glycerol levels. The magnitude of change was less for the SCPs than for glycerol levels. This indicates that it takes large changes in glycerol levels to produce small changes in SCPs.

Glucose and trehalose levels were similar to those seen in other aspects of this study. Both compounds displayed transitory fluctuations during maximum changes in glycogen and glycerol levels.

Table 12. Glycerol levels for reversibility experiment.
n = number of assays

Date	Glycerol levels in μmol/g female fleas				Glycerol levels in μmol/g male fleas			
	n	Mean	S.E.	Range	n	Mean	S.E.	Range
Day-zero [a]	2	389.6	52.4	352.5 - 426.6	2	396.6	37.1	370.4 - 422.8
Day-2 [b]	2	307.9	24.2	290.8 - 325.0	2	274.7	40.6	246.0 - 303.4
Day-4 [b]	2	208.6	2.0	207.2 - 210.0	2	257.3	7.9	251.7 - 262.9
Day-8 [b]	2	116.2	8.2	110.4 - 122.0	1	-----	---	---------
Recool [c]	2	313.0	9.2	306.0 - 319.0	2	286.0	8.7	279.8 - 292.1

a = -6 C mid-October

b = 15 C

c = -6 C for 18 days

Table 13. Glycogen levels for reversibility experiment.
n = number of assays

Date	Glycogen levels as μmol glucose/g wet wt. female fleas				Glycogen levels as μmol glucose/g wet wt. male fleas			
	n	Mean	S.E.	Range	n	Mean	S.E.	Range
Day-zero [a]	2	270.6	21.0	255.7 - 285.4	2	252.1	42.8	221.8 - 282.3
Day-2 [b]	2	270.1	5.5	266.2 - 274.0	3	285.7	25.7	256.8 - 306.2
Day-4 [b]	3	380.8	15.3	370.8 - 398.4	2	351.3	50.9	315.3 - 387.3
Day-8 [b]	1	427.3	---	--------	1	261.1	---	--------
Recool [c]	1	166.3	---	--------	1	138.7	---	--------

a = -6 C mid-October
b = 15 C
c = -6 C for 18 days

Table 14. Glucose levels for reversibility experiment. n = number of assays.

Date	Glucose levels in μmol/g female fleas				Glucose levels in μmol/g male fleas			
	n	Mean	S.E.	Range	n	Mean	S.E.	Range
Day-zero[a]	2	15.6	0.2	15.4 - 15.7	2	12.8	1.6	11.7 - 13.9
Day-2[b]	2	31.0	0.0	31.0 - 31.0	3	34.4	9.0	25.5 - 43.4
Day-4[b]	3	16.0	1.0	14.9 - 16.6	2	16.3	9.4	9.6 - 22.1
Day-8[b]	1	34.4	---	-------	1	22.7	---	-------
Recool[c]	1	13.2	---	-------	1	17.1	---	-------

a = -6 C mid-October
b = 15 C
c = -6 C for 18 days

Table 15. Trehalose levels for reversibility experiment.
n = number of assays

Date	Trehalose levels in μmol/g female fleas				Trehalose levels in μmol/g male fleas			
	n	Mean	S.E.	Range	n	Mean	S.E.	Range
Day-zero [a]	3	26.5	8.1	19.9 - 35.6	2	27.9	1.0	27.2 - 28.6
Day-2 [b]	3	16.4	2.2	14.3 - 18.6	2	16.8	8.3	10.9 - 22.6
Day-4 [b]	2	20.1	3.3	17.8 - 22.4	2	19.9	5.7	15.9 - 23.9
Day-8 [b]	1	19.0	--	-------	1	17.2	--	-------
Recool [c]	1	14.1	--	-------	-	---	--	-------

a = -6 C mid-October
b = 15 C
c = -6 C for 18 days

Table 16. Supercooling points for reversibility experiment.
n = number of fleas

Date	Supercooling points for females in degrees Celsius				Supercooling points for males in degrees Celsius			
	n	Mean	S.E.	Range	n	Mean	S.E.	Range
Day-zero [a]	8	-28.1	1.1	-26.4 - -29.5	8	-27.5	1.4	-26.1 - -30.4
Day-2 [b]	8	-27.7	0.5	-27.1 - -28.2	8	-26.6	1.8	-24.4 - -29.7
Day-4 [b]	7	-25.9	1.8	-24.4 - -29.5	6	-26.3	1.7	-26.4 - -27.4
Day-8 [b]	6	-25.7	1.9	-23.4 - -28.6	6	-27.4	1.6	-25.8 - -28.2
Recool [c]	6	-28.1	1.1	-27.1 - -30.1	6	-28.0	1.6	-25.8 - -29.7

a = -6 C mid-October
b = 15 C
c = -6 C for 18 days

Figure 23. Glycerol, glycogen and super cooling points for reversibility experiment. Male and female values pooled. Day-8 values for females only.
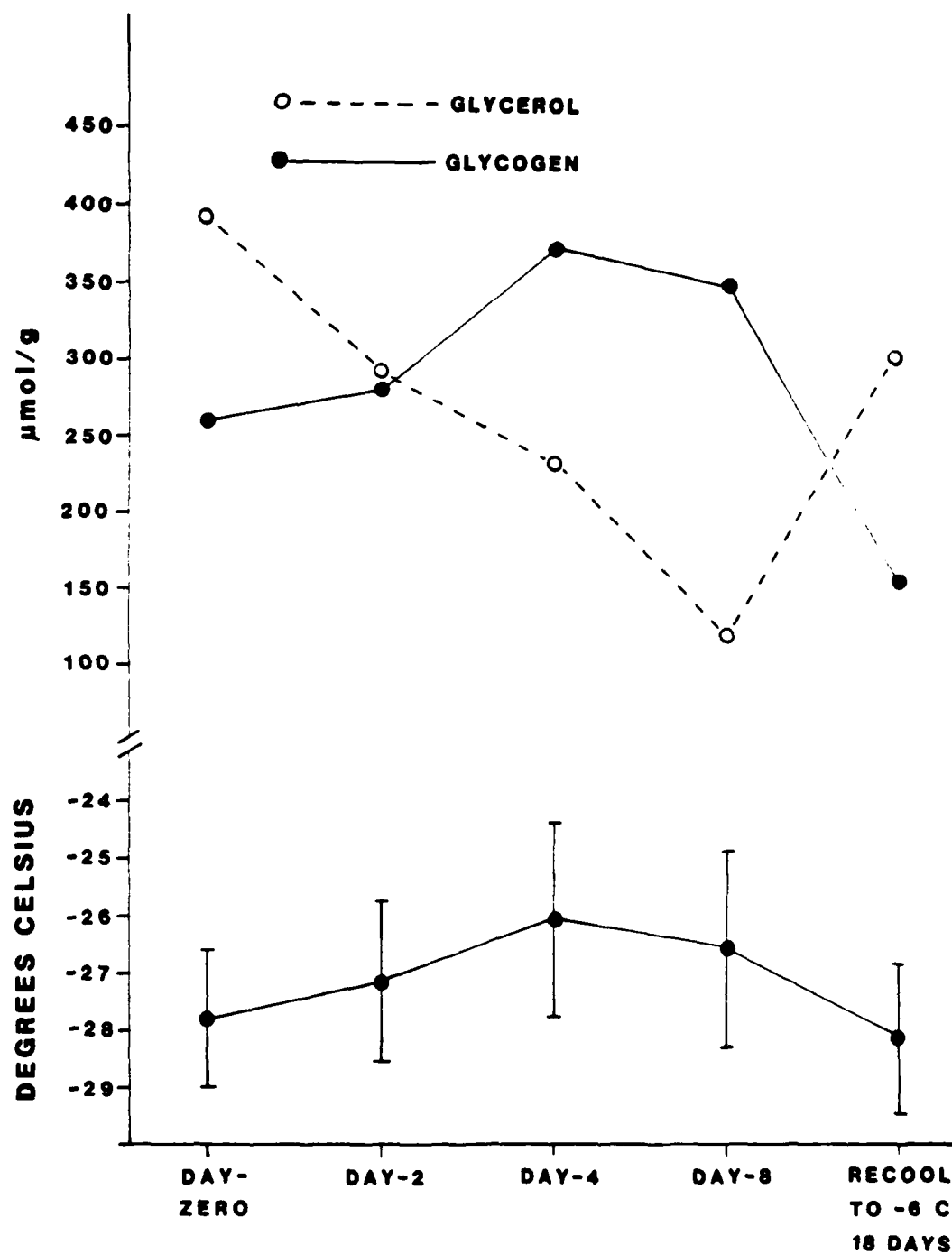
Survival Rates

Survival rates (Table 17 and Figure 24) from time zero through the cool-down (1 C) were high. Except for the sudden and unexplained die-off of ambient-held fleas in the winter of 1984-85, there was a steady decrease in survivorship after 1 C. In both freezer- and ambient-held regimens, female fleas tended to have slightly better survival rates than males, but these differences were not statistically significant at 5%.

Table 17. Survivorship of Ceratophyllus idius from time-zero through mid-May. Figures are combined from 1984-85 and 1985-86 seasons unless otherwise indicated.

Date	Ambient Survivors ♀	♂	Ambient Dead ♀	♂	Ambient % Survival ♀	♂	Freezer Survivors ♀	♂	Freezer Dead ♀	♂	Freezer % Survival ♀	♂
Time-zero	-	-	-	-	100.0	100.0	-	-	-	-	-	-
1 C 4-day	140	74	5	6	96.6*	92.5*	-	-	-	-	-	-
Mid-Oct	169	126	23	27	88.0	82.4	162	144	17	32	90.5	81.8
Mid-Nov	67	47	11	13	85.9*	78.3*	94	70	22	25	81.0	73.7
Mid-Dec	77	40	20	14	79.4	74.1	77	69	27	30	74.0	69.7
Mid-Jan-85	63	37	24	21	72.4	63.8	48	59	25	25	65.8	70.2
Mid-Feb-85	9	7	383[a]		4.0[a]		46	48	51	50	47.4	49.0
Mid-Feb-86	91	73	61	57	59.9	56.2	31	31	38	19	44.9	62.0
Mid-Mar-85	9	2	283[a]		3.7[a]		47	31	44	20	51.6	60.8
Mid-Mar-86	106	62	85	92	55.5	40.0	21	16	39	33	35.0	32.7
Mid-Apr-85	10	3	292[a]		4.3[a]		44	28	109	72	28.8	28.0
Mid-Apr-86	37	29	78	60	32.2	32.6	20	31	164	121	10.9	20.4
Mid-May-86	6	3	82	69	6.8	4.2	3	6	53	40	5.4	14.9

*1 C 4-day from 1985 only, Nov ambient from 1984 only, [a]male and female values pooled

Figure 24. Survivorship for ambient- and freezer-held
fleas for 1984-85, 1985-86.
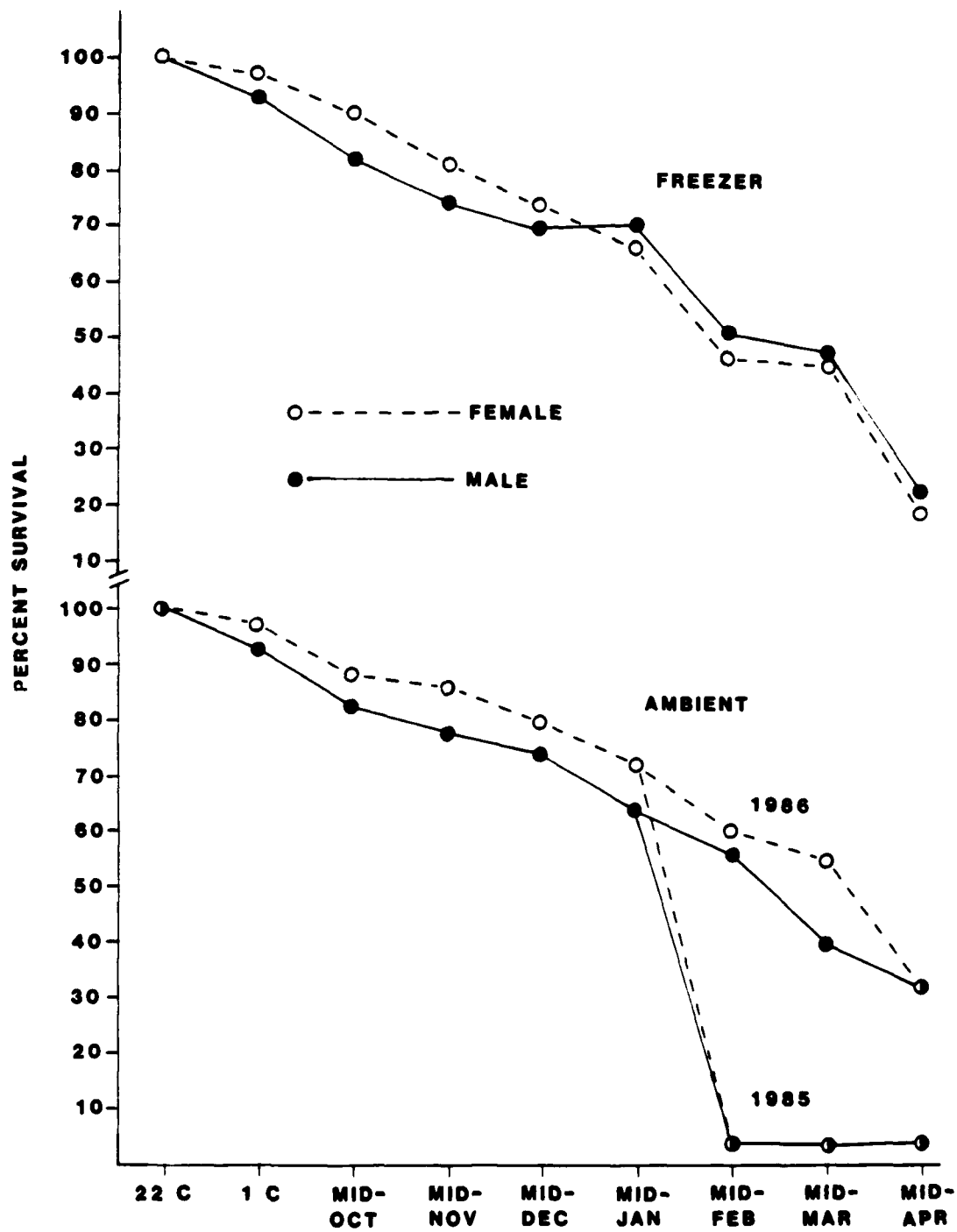
DISCUSSION

Host Specificity of Ceratophyllus idius

Reports in the literature indicate that C. idius is host specific for purple martins and tree swallows. Martin nests are shallow, 1-3 inches in depth, and are constructed primarily of sticks, mud and green leaves. The other common inhabitants of martin houses are English sparrows, Passer domesticus. Their nests consist of dry grasses which often fill the nest box except for a small cavity near the opening. One might speculate that C. idius would feed on sparrows in the absence of martins. However, without exception, fleas were never found in a house unless martin nests were present. There appeared to be a relationship between the number of martin nests and the number of fleas present in a house.

Induction and Variation in Glycerol Levels

Glycerol appears to be the main antifreeze synthesized by C. idius. The cool-down process in 1984 indicated that glycerol synthesis was induced at or prior to 10 C. The 1985 assays showed that induction had occurred prior to the collection of fleas. The unusual coolness of August and early September 1985, apparently triggered the induction process. Frequent night-time minima of 10 C or less during that time are comparable with induction temperatures reported for other insects. In the larval gall fly, Eurosta solidaginis, a temperature specific pattern relates to polyol levels. At 15 C glycerol is already present at 65%

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of the maximum level attainable between 5 and 0 C, and at
5 C sorbitol levels begin to rise (Storey, Baust and Storey
1981). In adult Nearctic carabid beetles, _Pterostichus_
brevicornis, glycerol synthesis was induced by exposure to
0 C for four days (Baust 1982). In the beech leaf mining
weevil, _Rhynchaenus_ _fagi_, induction of polyol synthesis oc-
curred at 15 C (Bale and Smith 1981). Therefore, tempera-
ture apparently is an important factor in the induction of
polyol synthesis. The actual triggering temperature varies
not only among species, but with geographical location
within a species. The triggering temperature for sorbitol
synthesis is 2-3 C lower in Minnesota than in Texas popu-
lations of _E_. _solidaginis_ (Baust and Lee 1982).

Once induced, glycerol synthesis in the flea does not
appear to proceed unabated or at constant rates. In 1985,
the time-zero to 10 C glycerol values did not differ sig-
nificantly, presumably because adequate levels of protec-
tion already had been produced. In contrast, glycerol
levels in fleas left at 10 C for 22 days showed a three-
fold rise while in those cooled to, and held at -6 C, had
levels that rose five-fold over the same period of time.
Therefore, it would appear that glycerol synthesis contin-
ues once it is induced as long as fleas are held at or
below the induction temperature. However, the difference
in glycerol levels between fleas held at 10 C for 22 days
and fleas cooled to -6 C indicates that there is regulation
which reflects both temporal and thermal aspects. The

regulatory sensitivity was also shown by the simulated mid-winter thaw experiment wherein glycerol levels dropped significantly when fleas were moved from -6 to 2 C for seven days. In the similar but more extreme warming to 15 C experiment, the decrease of glycerol levels was large (26%) within two days of warming. In E. solidaginis, the accumulation of sorbitol and trehalose also has both temporal and thermal components. Accumulation to maximum levels requires either progressive cooling to lower temperatures or an extended time at a triggering temperature equal to or less than 5 C (Baust and Lee 1982). For trehalose, large increases occurred within two weeks at -40 C, four weeks at -20 C, eight weeks at -5 C and 10 weeks at 5 C (Morrissey and Baust 1976). The same insect showed a 13% increase in glycerol levels after three days of cooling below triggering temperatures, and a 100% increase in sorbitol after 24 hours. In reversal experiments where gall fly larvae were rewarmed to 23 C, glycerol levels decreased slightly and sorbitol levels decreased 50% in 24 hours, and to precooldown levels by three days (Storey and Storey 1983a). When winter-hardened bark beetles, Pytho depressus, were rewarmed to 22 C, glycerol levels dropped 57% in 24 hours, and to zero in three days (Zachariassen 1977).

Fluctuations in glycerol levels of ambient-held fleas appear to be related to general and short-term temperature trends. The first evidence of this occurred in 1984 when 1 C fleas were placed on the roof. Unusually warm October

weather reduced glycerol levels by 49.2% from 1 C values.
Evidence from the two warm-up experiments indicated major
changes in glycerol levels following short-term changes in
temperature. In this context, the maximum temperatures for
two and four days prior to mid-monthly freeze-kills were
reexamined for the coldest portion of the winter;

mid-December	481.0 µmol/g:	2-day high =	-2.2	C
		4-day high =	-1.1	C
mid-January	375.6 µmol/g:	2-day high =	2.8	C
		4-day high =	2.8	C
mid-February	558.5 µmol/g:	2-day high =	-11.1	C
		4-day high =	-11.1	C

There does appear to be a pattern of fluctuating glycerol
levels that coincides with short-term ambient temperatures.

From experimental and ambient fluctations in tempera-
tures, it is apparent that $\underline{C}$. $\underline{idius}$ regulates glycerol
levels in direct response to temperature changes. The
sensitivity of the system suggests a second trigger close
to 0 C that regulates increased rates of glycerol synthesis
when temperatures fall and reduced levels when they rise.
In any event, $\underline{C}$. $\underline{idius}$ possesses a rapid and effective
mechanism for regulating glycerol levels, thus insuring
adequate cryoprotection during the large temperature fluc-
tuations common in the spring and fall, and also the mid-
winter thaws that occur in eastern North Dakota.

Purple martin fleas are not unique in this pattern of
temperature dependence/reversibility of glycerol levels.

A number of insects occupying natural habitats have been analyzed in this respect. Hansen, Viyk and Luyk (1982) reported that cold-hardiness in the bark beetle, Ips typographus, changed in the course of the season in response to changes in mean air temperature. In the Nearctic carabid beetle, P. brevicornis, mean glycerol levels corresponded with seasonal variations in daily high-low temperatures (Baust 1982). Variations in polyol concentrations as measured by hemolymph osmolality showed a nearly two-fold difference in the bark beetle, Ips acuminatus, between those hibernating on the sun-exposed side and those on the colder north side of pine trees (Gehrken and Zachariassen 1977). The SCPs of these beetles were reported to change in response to variations in ambient temperatures from November through April and the SCPs paralleled the amount of polyol present in the insects (Gehrken 1984).

Glycerol Synthesis

The identification of the source for glycerol synthesis provides a clue to possible enzymatic mechanism(s) controlling its accumulation as an antifreeze. Insects utilize different precursors because of different metabolic pathways (Chino 1957; Asahina 1969; Baust and Miller 1972). Wood and Nordin (1976) proposed two possible sources for glycerol accumulation in the arctic blowfly, Protophormia terranovae; (1) lipid glycerol from fat body tissue, and/or (2) hexose units derived from glycogen or stored dietary carbohydrate. A direct cold activation of

lipase in the fat body leading to release of free glycerol was proposed by Baust and Miller (1972). In diapausing eggs of the silkworm, Bombyx mori, isotope [^{14}C] studies showed that about 1/3 of the free glycerol pool came from lipids (Yaginuma and Yamashita 1980). However, the same study revealed that reduced glycogen radioactivity corresponded with increased radioactivity in glycerol and sorbitol. Direct or indirect evidence of glycogen serving as a major source for polyols has been proposed for a variety of insects (Chino 1958; Wyatt and Meyer 1959; Mansingh and Smallman 1972; Ziegler and Wyatt 1975; Wood and Nordin 1976; Ring 1982; Pigage and Larson 1983; Storey and Storey 1983b) The results reported in this dissertation support the view that glycogen is the primary precurser for glycerol. It should be noted that lipid levels in C. idius remained stable throughout the winter (Kuhn unpublished data); therefore, it appears that fats are not the major source for glycerol production.

Other Antifreezes

In some insects, sorbitol and/or trehalose are primary, if not the only antifreeze(s)/cryoprotectant(s) produced, and glucose may also play a minor role. In C. idius, sorbitol levels remained barely detectable even in the extreme cold of mid-winter; therefore, sorbitol cannot be considered to be a factor in the cold-hardiness of this flea. Minor fluctuations probably reflected variations inherent in the assay methodology. Trehalose levels also

were low. The transitory two-fold increase at 13 C, and
the three-fold rise in mid-March ambients both precede
major changes in glycogen levels. There may be some antic-
ipatory metabolic processes occurring prior to glycerol
synthesis or degradation that is reflected in these elevat-
ed trehalose levels. As with sorbitol, trehalose cannot be
considered a factor in cold-hardiness of C. idius. Glucose
also is not a primary protective agent despite a 3-4 fold
rise in mid-October. It seems probable that this increased
level of glucose reflects the high rate of glycogen degrada-
tion occurring at the time.

Supercooling points

The low supercooling points for the time zero fleas
are thought to be due to a combination of three factors.
The most important of these is an empty gut in the absence
of a host. This would eliminate INAs from the gut. Salt
(1953) found that cold-hardiness of feeding insect larvae
compared with non-feeding larvae was reduced by the pres-
ence of food in the digestive tract. SCPs dropped from
-10.3 to -23 C for feeding versus non-feeding larvae of the
pale western cutworm, Agrostis orthogonia, while larvae of
the Mediterranean flour moth, Ephestia kuhniella, dropped
from -11.0 to -20.5 C. Krunic (1971) reported that feeding
larvae of the solitary bee, Megachile rotundata, were less
hardy than non-feeding stages. Krunic and Radovic (1974)
concluded that an increased SCC of 20 C from the larval to
the prepupal stage was due to evacuation of the gut.

A second factor that may have contributed to low time-zero SCPs is the fact that the fleas were held at 22 C for about three weeks. This undoubtedly resulted in partial dehydration and increased solute concentrations, thus lowering the SCPs.

The third factor is that 1985 fleas already had a large amount of glycerol by time they were collected. Although this level probably had decreased by the time the SCPs were determined, some glycerol may still have been present that contributed to lowered SCPs.

In $\underline{C}$. $\underline{idius}$, lowered SCPs corresponded with increased glycerol levels. This was readily apparent in the reversibility experiment; therefore, it appears that SCPs can be used as an indicator of the overall cold-hardiness of $\underline{C}$. $\underline{idius}$.

The SCPs for the fleas were compared with those of insects known to be either freeze-tolerant or freeze-susceptible. SCPs for six cold-hardened, freeze-tolerant species ranged from -6.3 to -14 C (Zachariassen 1985), whereas those for 72 species of adult, cold-hardened, freeze-sensitive insects ranged from -5.6 to -38.9 C (Sømme 1982). Compared to these values it appears that purple martin fleas with a cold-hardened mean SCP of -29.9 C are freeze-susceptible. However, there are some troublesome aspects to this interpretation. The mid-February (1986) SCPs for ambient-held fleas ranged from -26.7 to -32.0 C. These specimens had a few days earlier

survived temperatures of -29.4 C. If indeed C. idius is
freeze-susceptible, how did those with SCPs above -29.4 C
survive? Miller (1978) demonstrated that the adult
tenebrionid beetle, Upis ceramboides, requires cooling
rates well below 1 C/min to achieve its full cold-hardiness
capability; therefore, the actual SCP produced by ambient
conditions may be lower than that determined in the labora-
tory. Perhaps C. idius may be one of those rare insects
that is freeze-tolerant, but has a low SCP. Such a cond-
ition is known for the bark beetle, Pytho deplanatus, which
can attain an SCP of -54 C (Ring 1980).

Flea Survival

A small but adequate percentage of ambient-held fleas
survived to mid-April, ensuring that successful reinfes-
tation of martins would occur. The fact that some fleas
survived until mid-May implies that even if the birds were
late in returning, there still would be viable fleas to in-
sure continuation of the local populations.

Biological Timing Mechanism

In some insects, biological timing mechanisms have been
shown to play a role in inducing cold-hardiness. Photo-
period is one of the governing factors for cold-hardening
in the bark beetle, I. typographus (Schopf 1985). Horwath
and Duman (1982) reported that the induction of THF in the
larvae of the bark beetle, Dendroides canadensis, is de-
pendent on a critical light:dark period of 8:16. This in-
sect overwinters in dead tree trunks. Wherever thermally

buffered microhabitats fail to provide precise triggering of polyol synthesis, photic cues may be important for the induction of cold-hardiness (Baust 1982).

Since C. idius occupies a harsh thermal habitat, it is doubtful that photoperiod plays a role in establishing cold-hardiness. Equally doubtful is the hypothesis of an an innate timing mechanism for reducing glycerol levels in the spring. My results from freezer-held fleas do not indicate the presence of such a timing mechanism.

Overall Conclusion

It appears that C. idius is well adapted to survival in eastern North Dakota with its sensitive temperature dependent mechanism for controlling glycerol levels. This mechanism can respond to the wide fluctuations in temperatures common in the fall, spring and mid-winter thaws. As a result, a portion of the adult flea population is able to successfully overwinter and reinfest purple martins upon their return in April.

APPENDICES

Appendix I

Data Collection Sheet For Glucose/Glycogen Assays

	S 1	S 2	S 3	♀1	♀2	♀3	♂1	♂2	♂3
O.D.									
AVE.									

$\dfrac{\text{O.D. STD}}{\mu g/ml\ \text{STD}} = \dfrac{\text{O.D. Samp.}}{X}$	X	Total μg (x 8-Glucose) (x 4.6-Glycogen)	μg/mg flea	μm/gm flea (÷ mw=180)	μm/mg flea (x 1000)
♀					
♂					

Appendix II

Data Collection Sheet For Trehalose Assays

	Blank	S 1	S 2	S 3	♀1	♀2	♀3	♂1	♂2	♂3
O.D.										
Ave.										
- Blk	——									

$\dfrac{\text{O.D. STD}}{\text{µg/ml STD}} = \dfrac{\text{O.D. Sample}}{X}$	Total µg (x 23.5)	µg/mg flea	µm/mg flea (÷ 378)	µm/g flea (x 1000)
X				
♀				
♂				

Appendix III

Data Collection Sheet For Glycerol Assays

Date: _____ Assay: Glycerol Regime: _____ Grind #: _____

	Blank	S 1	S 2	♀ 1	♀ 2	♀ 3	♂ 1	♂ 2	♂ 3
D/D H_2O	0.80 ml	0.60	0.60	0.70	———————————————————————————>				
Sol. 1	0.40 ml			———————————————————————————————>					
Sample/STD	—— ml	0.20	0.20	0.10	———————————————————————————>				
Sol. 2	0.004 ml			———————————————————————————————>					
Mix, Read T_1 A_1 after	———	——	——	——	——	——	——	——	——
7 min A_1	———	——	——	——	——	——	——	——	——
Add Sol. 3	0.004 ml	———>							
Read T_2 T_2 after	———	——	——	——	——	——	——	——	——
20 min A_2	———	——	——	——	——	——	——	——	——
Change $\triangle$									
$\triangle_S - \triangle_B$									
Mean change in O.D.									

$$\frac{X\triangle STD}{\mu g/STD/ml} = \frac{X\triangle Sample}{X}$$

X = μg/ml		
Total μg (x 24.16)		
μg/mg flea		
μmol/mg flea (÷MW) 92		
x 1000 μmol/g flea		

	# Fleas	Paper + Fleas	Wt. Paper	Flea Wt.	Ave.
♀					
♂					

Appendix IV

Data Collection Sheet For Sorbitol Assays

Date: _____   Assay: Sorbitol   Regime: _____   Grind #: _____

	Blank	S 1 S 2	♀ 1 ♀ 2 ♀ 3	♂ 1 ♂ 2 ♂ 3
D/D H_2O	0.80 ml	0.44 0.44	0.44 ─────────────────────>
Sol. 1	0.24 ml	0.24 ───────────────────────────>
Sol. 2	0.08 ml	────────────────────────────────>
Sol. 3	0.08 ml	────────────────────────────────>
STD/Sample	_____	0.36 ml ─────────────────────>
Mix, Read T_1 A_1 after 2 min A_1				
Add Sol. 4	0.02u ml	───────────────────────────────>
Read T_2 T_2 after 30 min A_2				
Change $\triangle$				
$\triangle_S - \triangle_B$				
Mean change in O.D.				

$$\frac{X \triangle STD}{\mu g/STD/ml} = \frac{X \triangle Sample}{X}$$

$\frac{X \triangle STD}{\mu g/STD/ml} = \frac{X \triangle Sample}{X}$		
X = μg/ml		
Total μg (x 5.56)		
μg/mg flea		
μmol/mg flea (÷MW) ÷ 182		
x 1000 μmol/g flea		

	# Fleas	Paper + Fleas	Wt. Paper	Flea Wt.	Ave.
♀					
♂					

Appendix V

Calculations Used To Determine Micromoles Of Glucose/Gram Of Flea

1. Glucose concentration in the assay tube (X) was multiplied by a factor of 8 to determine total g in the homogenized sample.

This factor was based on (1) the volume of homogenized sample (2.0 ml), (2) the amount of sample assayed (0.3 ml) and (3) the total volume of solution in the assay tube (1.2 ml).

$$\frac{2.0 \times 1.2}{0.3}$$

Therefore, using the above volumes, the factor = 8 (i.e., 12.5% of the liberated glucose was present in each assay tube). For glycerol, sorbitol and trehalose assays, the factor varied due to differences in one or more of the three variables listed above.

2. Total µg of glucose was divided by the weight (mg) of fleas to give µg glucose per mg of flea.

3. The result of step 2 divided by the molecular weight of glucose (180) gave µmol glucose per mg of flea.

4. The result of step 3 (µmol/mg) multiplied by 1000 gave µmol/g of flea.

Appendix VI

Preparation And Concentrations Of Reagents For Glycogen Hydrolysis

Amyloglucosidase was purchased from Boehringer Mannheim Biochemicals.

The following steps were taken from Keppler and Decker (1974).

All solutions were prepared using deionized/distilled (D/D) water.

1. Perchloric acid (0.6N):
   Dilute 5.2 ml 70% $HClO_4$ with D/D water to 100 ml.

2. Potassium hydrogen carbonate (1M):
   Dissolve 2 g potassium hydrogen carbonate in D/D water and make up to 20 ml.

3. Acetate buffer (0.2M; pH 4.8):
   Dissolve 4.8 ml 96% acetic acid and 16.17 g hydrated sodium acetate ($CH_3COONa \cdot 3H_2O$) in D/D water and make up to 1000 ml. Check the pH with a glass electrode.

4. Amyloglucosidase (100 mg/10 ml):
   Dilute 100 mg of enzyme protein in 90 ml acetate buffer.

Amyloglucosidase can be stored deep-frozen without any appreciable loss of activity.

Appendix VII

Contents Of Solutions For Glycerol And Sorbitol Analyses

Solutions (Sol.) were from Boehringer Mannheim Biochemicals enzymatic food analysis kits. Contents are as follows:

Glycerol

Sol. 1: glycylglycine buffer, NADH, ATP, PEP and magnesium sulfate stabilizers

Sol. 2: pyruvate kinase and lactate dehydrogenase

Sol. 3: glycerokinase

Sorbitol

Sol. 1: potassium phosphate/trethanolamine buffer and Triton X 100

Sol. 2: diaphorase, B-NAD and stabilizers

Sol. 3: iodonitro tetrazolium chloride (INT)

Sol. 4: sorbitol dehydrogenase (SDH)

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